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PHOTORECEPTION

BY

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PHOTORECEPTION*

Consulting Editor
JEROME J. WOLKEN

CONTENTS

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INTRODUCTORY REMARKS

By Albert S. Gordon

The New York Academy of Sciences and the Department of Biology, Graduate School of Arts and Science, New York University, New York, N. Y.

The monthly meetings of the various sections of The New York Academy of Sciences often serve as a testing ground for subjects that show potential promise of expansion into conferences. The conference of which this monograph is the result arose as an outgrowth of a meeting of the Section of Biology held in 1957, during which Jerome Wolken of the University of Pittsburgh, Pittsburgh, Pa., delivered a lecture entitled "A Comparative Study of Photoreceptors." His presentation was so well received and the ensuing discussion so lively that Ross F. Nigrelli, then president of the Academy, prevailed on him to organize a conference on photoreception, an invitation he was kind enough to accept.

Our understanding of the mechanisms underlying photoreception has advanced greatly in recent years. Greater knowledge of the structure of photoreceptor units, more penetrating analysis of visual pigment chemistry, and greater exploration of the physiological implications have all aided materially in augmenting our basic information in photoreception. This monograph treats various aspects of these problems.

Once again, as in previous monographs published by the Academy, the importance of the phylogenetic approach as a means of uncovering basic biological principles is highlighted. Experiments involving a wide diversity of living forms, from bacteria to bees to man, and even some plants, are presented. The general similarity of the pattern emerging from these phylogenetic considerations should help to dispel the erroneous concept, still held by some, that the understanding of biological phenomena in humans can come only from experiments performed on man himself.

Part I. Photoreceptor Structures

STUDIES OF PHOTORECEPTOR STRUCTURES*

By Jerome J. Wolken

School of Medicine, University of Pittsburgh, and the Eye and Ear Hospital, Pittsburgh, Pa.

The stimulation of living organisms by light is mediated through photosensitive pigments within photoreceptors. The responses are known as photokinesis, phototaxis, photosynthesis, and vision. The photoreceptors in photosynthetic bacteria, protozoa, algae, and the higher plants are chromatophores, plastids, and chloroplasts; in certain flagellated protozoa, the stigma, or eyespot; in the invertebrates, the sensory cells, ocelli, ommatidia, rhabdomeres, and retinal cells; and, in the vertebrates, the retinal rods and cones of the eye. The terminology applied to these photoreceptors is intended to indicate something of their phylogenetic as well as their physical structure.

A comparative study of plant and animal photoreceptor structures by means of electron microscopy and physical biochemical methods is now in progress in this laboratory. This paper presents a part of the research done by my associates and myself on the animal photoreceptors. The results of our studies of plant photoreceptors have already been made available,[1-6] as have reviews by Thomas,[7] Mühlethaler,[8] Granick,[9] and Rabinowitch.[10]

The major tool in these researches has been the electron microscope, which calls to mind the fact that Anton van Leeuwenhoek is said to have first examined a retina with his crude microscope in 1674, and to have described the "globules" in it. It is also of historical interest, since osmium tetroxide (OsO_4), referred to as osmic acid, is still our best fixing agent for electron microscopy, that in 1866 Schultze used it when he described two different receptor cells of the retina, the rods and the cones.

Many of the photoreceptors—the chloroplasts and retinal rods—can be isolated and their size and some of their physical and chemical properties determined by light, polarizing, phase, and interference microscopy.[11, 12] The X-ray microscope with its present limited use has added no additional structural information. Fortunately, the electron microscope, together with current techniques in tissue fixation, in embedding, and particularly in thin-sectioning, has provided us with another means for studying the photoreceptors. The kind of morphologic information this yields, together with that of other analytical methods, gives considerable hope for a molecular picture of a photoreceptor.

General photosensitivity is widespread throughout the invertebrate phyla. Eyespots, sensory cells, compound ocelli and compound eyes are found

* The research for this paper was supported in part by Research Grant G-199 (C5) from the National Council to Combat Blindness, Inc., New York, N. Y.; in part by Research Grant B-397 (C4) from the National Institute of Neurological Diseases and Blindness, Public Health Service, Bethesda, Md.; and in part by a grant from the McClintic Endowment, Pittsburgh, Pa.

among annelids, mollusks, and arthropods, in each instance with differences in gross physical organization. Since the invertebrates possess the greatest variety of photoreceptors and are structurally least understood, I shall describe several that we have studied: the stigma or eyespot of the protozoan flagellate *Euglena;* the sensory cells of the platyhelminth *Planaria;* the ommatidia of an arthropod insect *Drosophila;* and the retinal cells of the mollusk cephalopods, *Octopus* and *Sepia.** The invertebrate photoreceptors have been reviewed and discussed recently by L. J. and M. J. Milne,[13] Wulff,[14] and de Vries.[15]

Protozoa

Stigma. The stigma or eyespot is a photoreceptor for light perception. In the flagellate *Euglena* it is in the anterior part of the organism and appears in the light microscope as an orange-red area near the flagella. In the electron microscope the eyespot area for *Euglena gracilis* can be seen to be about 2 μ in diameter and 3 μ in length, and to consist of between 40 and 50 packed granules (rods) ranging from 100 to 300 mμ in diameter, which are embedded in a matrix.[2, 5] The eyespot is intimately linked to the flagella at its base. It is believed that the eyespot and flagella act as a unit in phototactic response directing the organism toward light. On the basis of the action spectra absorption peak at 465 mμ, the rate of swimming, and the cross-sectional area of the eyespot, the threshold energy needed to produce the response was calculated to be $\sim 3 \times 10^{-11}$ ergs per stigma.

Willmer[16] suggests that structurally the most interesting feature in the development of the rods and cones is the flagellumlike fibers that connect the outer and inner segments. Fauré-Fremiet and Rouiller[17] have suggested, as a result of their studies of the chrysomonad *Chromulina*, that the second internal flagellum associated with the stigma possesses a fine structure similar to that of the outer segments of the vertebrate retinal rods and cones. Red variants of *Euglena* contain astaxanthin, which is an animal pigment, and some action spectra indicate that this may be the phototactic pigment. Other experiments indicate that β-carotene may also be present in the eyespot. It has been suggested that the eyespot may contain at least two pigments. However, as yet no one has isolated astaxanthin, β-carotene, or any other photosensitive pigment, from the stigma of green *Euglena.* If, as suggested, all the eyespot granules were covered with pigment (a carotenoid) as a monolayer or double layer, there would be $\sim 1 \times 10^6$ pigment molecules.[5, 18] The orientation of the eyespot granules at the base of the flagella in *E. granulata* is illustrated in FIGURE 1.

Platyhelminths

Sensory Cell. In the flatworm *Planaria*, the two eyes are sense organs each consisting of pigment granules and sensory cells. The ends of the sen-

* I am indebted to the Zoological Research Station, Naples, Italy, for supplying me with *Octopus* and *Sepia* during my stay in Naples on a fellowship from the National Council to Combat Blindness in the summer of 1957. I am also indebted to the SeaQuarium, Miami, Fla., for supplying me with eyes of the baby black pilot whale, *Globicephala macrorhyncha.*

sory cells continue as nerves that enter the brain. The pigment shades the sensory cells from light in all directions but one, thus enabling the animal to respond in a negative way to the direction of light. The sensory cell (retinal structure) is behind the dense pigment granules and can be seen easily in the electron microscope. The sensory cell retinal lamellar structure in *Planaria* is illustrated in FIGURE 2. It is 5 μ in diameter, and has a somewhat variable length, which is difficult to measure, of approximately 35 μ. The retinal structure consists of dense plates or discs, which are double-membraned.

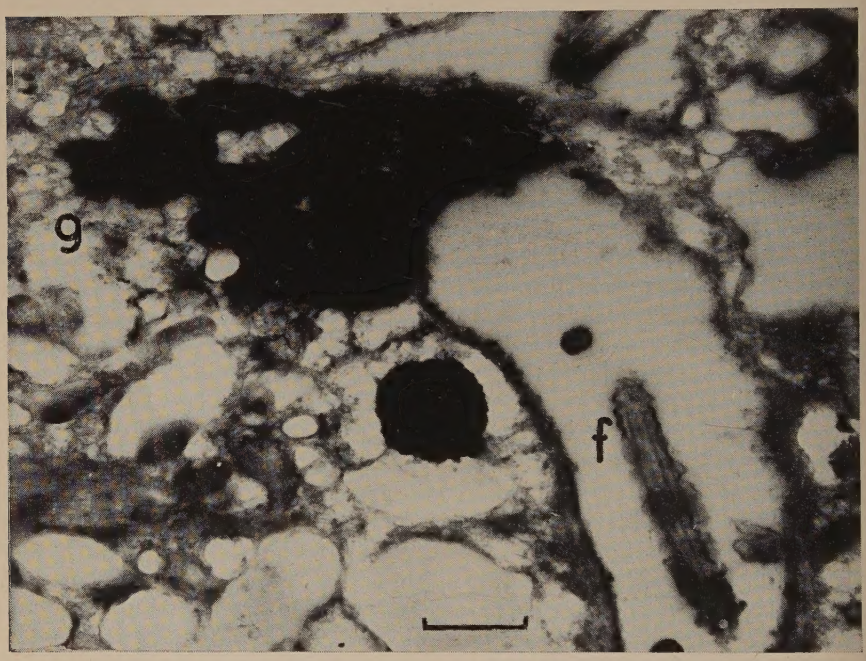

FIGURE 1. Electron micrograph of the stigma of *Euglena granulata* showing the eyespot granules *g* and the flagellum *f*. ×10,400. Other eyespot electron micrographs are available.[2, 5]

There are between 8 and 10 dense layers, or from 16 to 20 dense surfaces, per micron, and the total thickness of these layers is ~400 Å. Since they are double-membraned, each edge is ~100 Å in thickness. In structure the plates resemble the outer segments of the vertebrate retinal rods and cones. Little is known of the photosensitive pigment within these receptors. Pirenne and Marriot[19] have studied the eye of the planarian aquatic flatworm *Dendrocoelum lacteum* to determine its effectiveness or action spectrum. They found two main absorption peaks, one at 510 mμ, which they designated as probably a rhodopsin, and another in the ultraviolet at 370 mμ. They have calculated from their experiments that the absolute sensitivity or energy is $\sim 15 \times 10^{-9}$ ergs per eye per second.

Arthropods

Ommatidia. The compound eye of the insect consists of ommatidia, each ommatidium containing retinular cells, of which the differentiated part is the rhabdomere. Since the earliest investigations, the rhabdomeres have been considered the "light trapping" area in which the visual process is initiated. The eye of *Drosophila* is composed of approximately 700 ommatidia; each ommatidium contains seven retinular cells radially arranged to form a cylinder, and is 17 μ in diameter, with a length varying from

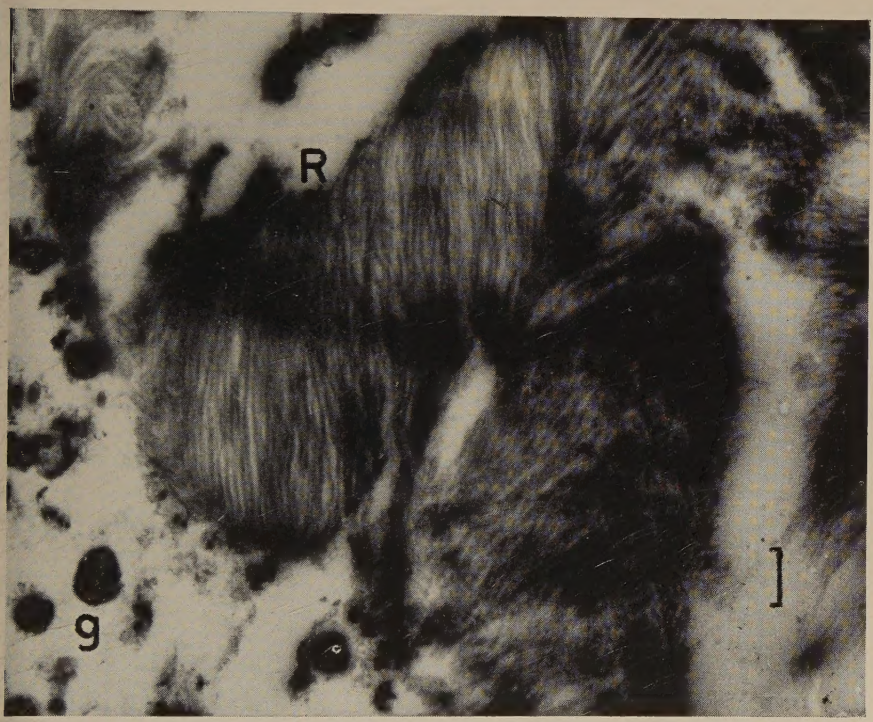

FIGURE 2. Electron micrograph of the sensory cell of *Planaria* showing the retinal element *R*, and the pigment granules *g*. ×6400.

70 to 125 μ. Electron microscopic studies indicate that the rhabdomeres are structurally packed rods or tubes, the thickness of the dense edges averaging 120 Å and the interspaces varying from 200 to 400 Å.[20, 21]

It has been shown that the rhabdomere of the insect is analogous to the retinal rod and cone. Moreover, by electron microscopy, Fernández-Morán,[22] Goldsmith and Philpott,[23] and Miller[24] have shown, not only that, in other insects, as well as in a crustacean, the retinular cells are in a definite arrangement within the ommatidium, but also that the rhabdomeres of the housefly, dragonfly, honeybee, spider, and *Limulus* are packed rods. It has been tentatively suggested that both the arrangement of the rhabdomeres

within the ommatidium and their fine structure may be related to the analysis of polarized light.[21-24] No visual pigment has been isolated as yet from the rhabdomere of an insect.* Although in mutants having three eye colors it was found that the action spectrum is indicative of a pigment absorbing at 508 mμ, we have not isolated this pigment from *Drosophila* rhabdomeres.[18, 20] However, Wald and Hubbard[25] have recently isolated a pigment from the rhabdomeres of the eyes of a crustacean, the lobster, which they designated as visual lobster rhodopsin, since it has the prosthetic group, retinene$_1$.

Mollusks

Retina. There is a remarkable resemblance in physical organization between the eyes of the cephalopod mollusks and those of the vertebrates. By scrutinizing the retinal structure of the eyes of *Octopus* and *Sepia* by electron microscopy, I have found that the arrangement of the retinal cells is very much like that in the arthropod ommatidia, with retinular cells and rhabdomeres. The similarity in structure to that of the insect[20, 21] and to *Limulus* is most striking.[24] In both *Octopus* and *Sepia* the fine structure of each retinal unit (the rhabdomere) is composed of densely packed rods or tubes averaging 1 μ in diameter with interlamellar spacings of 200 Å in thickness; there are about 20 such rods per micron. FIGURES 3, 4, and 5 show the retinal elements, the rhabdomeres. For comparison with the rhabdomere of the insect *Drosophila*, see FIGURE 6.

Vertebrates

Retinal rods. Earlier microscopic studies by Schmidt[11] had already indicated that the retinal rod is composed of alternate layers of lipid and protein. All the vertebrate photoreceptors studied recently—the retinal rods of the frog, chicken, guinea pig, rabbit, perch, whale, cow, and monkey—appear in the electron microscope as double-membraned structures resembling piled-up plates or disks between 100 and 200 Å in thickness with less dense interspaces of from 200 to 500 Å.[4, 18, 26-28] Techniques in low-angle X-ray diffraction using OsO_4 for fixation have corroborated the electron microscopy, showing similar spacing of ~200 Å.[29] The outer segments of the rods of frog, chicken, cow, and monkey are illustrated in FIGURES 7, 8, 9, and 10.

On the basis of our microscopic studies we can now distinguish three kinds of structurally built photoreceptors: granules, either isolated or more generally packed as a mosaic; a lamellar packing of double-membraned disks or plates; and tightly packed rods or tubes. Although there are many differences in the macrostructure of the photoreceptors, there is a great similarity in their ultramicrostructure. This is schematically represented in FIGURE 11. At present we have no analytical tool adequate for determining the exact orientation of the pigment or the other molecules within

* Elsewhere in this monograph T. H. Goldsmith has reported the presence in extracts fo honeybee heads of a photosensitive pigment with an absorption maximum at 440 mμ. My colleague J. M. Bowness and I have since isolated a photosensitive pigment from the housefly with an absorption maximum at 437 mμ. (Abstracts 134th American Chemical Society, 29C, 1958).

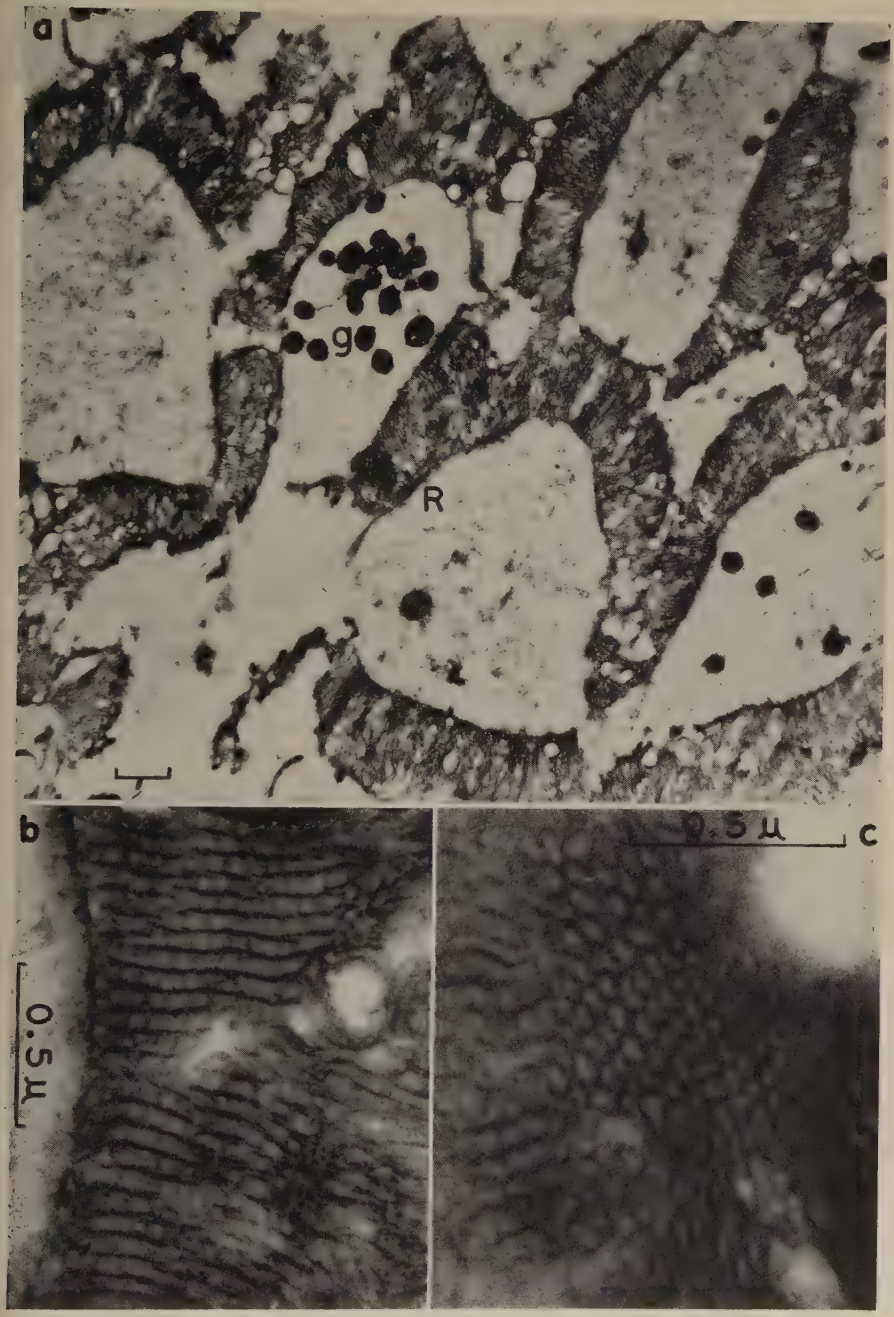

FIGURE 3. (a) Electron micrograph of the retinal unit of *Octopus* showing the retinal structures *R*, and the pigment granules *g*, ×7,150; (b) enlarged area of *R*, ×40,000; and (c) another enlarged area of *R*, ×56,000.

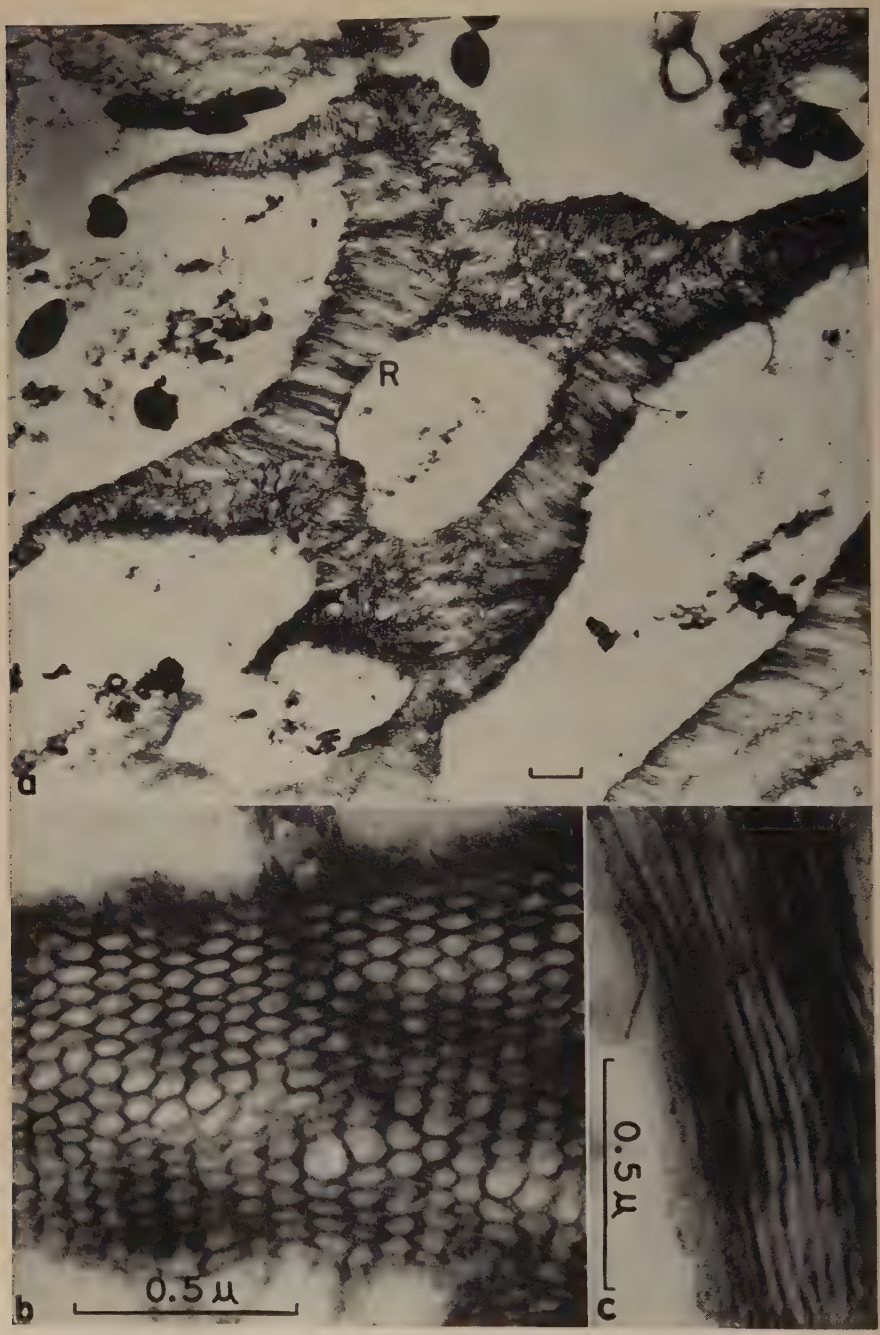

FIGURE 4. (a) Electron micrograph of the retinal unit of *Sepia* showing the retinal structures *R*, ×6500; (b) enlarged area of *R*, ×59,000; and (c) another enlarged area of *R*, ×59,000.

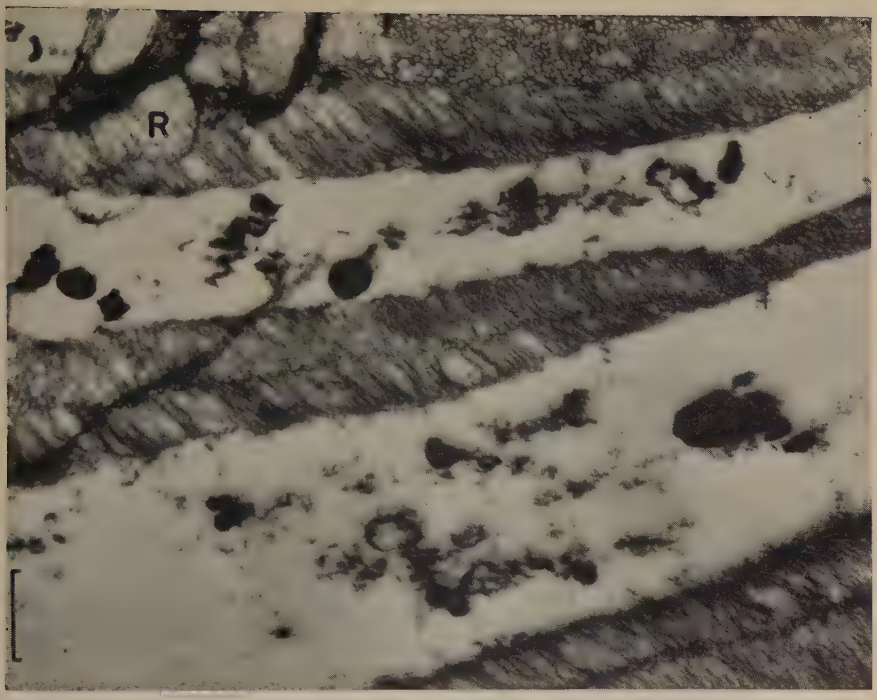

FIGURE 5. Electron micrograph of the retinal unit of *Sepia* showing a longitudinal section of the retinal structures *R*. ×12,000.

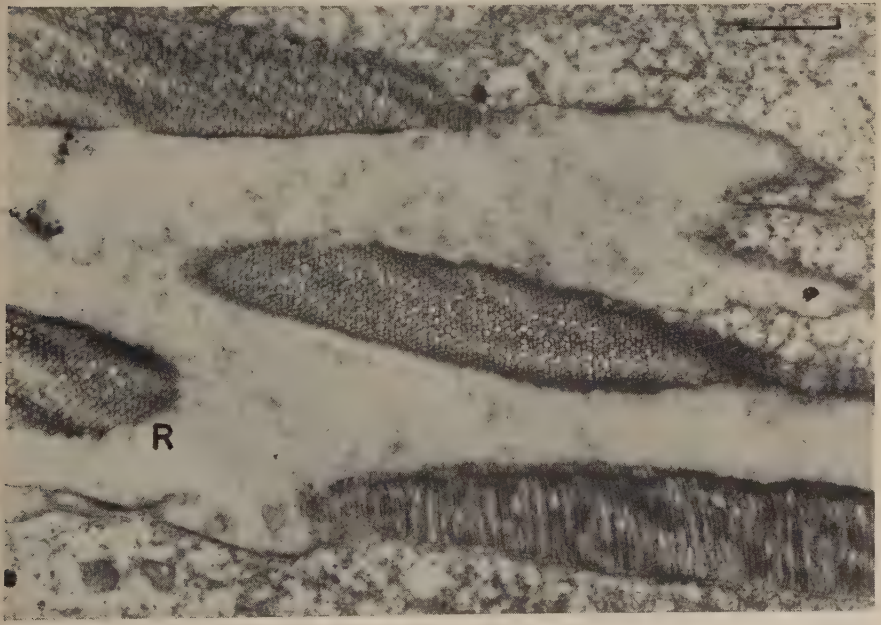

FIGURE 6. Electron micrograph of the ommatidia of *Drosophila melanogaster* showing an oblique section of the rhabdomeres *R*. ×13,000. Other electron micrographs are available.[20, 21]

these structures. The precise locations of the pigment, lipid, and protein within the photoreceptor can only be assumed from the chemical reactions of the biological material with the fixing agent and the various stains. Some experimental evidence indicates that in the chloroplasts the pigment molecules most probably reside in the (osmium-fixed) dense bands.[7, 30] Also, there are apparently just sufficient pigment molecules in each of the (dense) interfaces to cover all the available dense surfaces.[2-4, 20] The photoreceptor

FIGURE 7. Electron micrograph of the retinal rod of a frog, longitudinal section. ×12,000.

geometry (length, diameter, thickness, and number of dense layers) as determined from the electron micrographs (TABLE 1) can be used to calculate either the cross-sectional area of the pigment molecule or its complex, providing the pigment concentration of the specific photoreception is known, or can be calculated or experimentally determined. The average number of pigment molecules per receptor for all the plant and animal photoreceptors studied to date is reported to be between 1×10^6 and 1×10^9.

The dense layer consists of a double layer containing lipids and lipoprotein. The pigment molecules are believed to be oriented within these dense layers. This arrangement is shown in FIGURE 12. The maximum cross-sectional area

FIGURE 8. Electron micrograph of the retinal rod of a chicken, longitudinal section. ×13,000.

A, which would be associated with each macromolecule and therefore with each pigment molecule, can be calculated from

$$A = \frac{\pi D^2}{4P}$$

where D is the diameter of the photoreceptor and P is the number of pigment molecules in a single monolayer. A, which is associated with each macromolecule and therefore with each pigment molecule, can be derived from the above equation, where P is replaced by $N/2n$, in which N is the pigment concentration in molecules per photoreceptor and n is the number of dense layers per photoreceptor, as follows:

$$A = \frac{\pi D^2 n}{2N}$$

For example, for cattle and frog rhodopsin the cross-sectional area has been calculated to be 2500 Å^2, and the diameter of the molecule to be ~50 Å. This seems to be about the right order of magnitude for the rhodopsin molecule.[31]

FIGURE 9. Electron micrograph of the retinal rod of a cow, longitudinal section (note how plates are stacked). ×31,000.

The pigment macromolecular weight M of the complex can also be calculated from

$$M = \frac{\pi D^2 T s L n}{4N}$$

where D is the diameter of the photoreceptor; T is the thickness of the dense layers; s is taken as 1.3, the density of the protein; L is 6×10^{23}, Avogadro's number; n is the number of dense layers; and N is the number of pigment molecules per photoreceptor. This weight has been calculated for the rhodopsin of the frog and the cow as 60,000 and 40,000, respectively.[4, 18]

With respect to the plant photoreceptors, considerable information on their chemical composition, pigments, and structures is available. The electron microscope indicates a lamellar structure for a variety of chloroplasts from

FIGURE 10. Electron micrograph of the retinal rod of a rhesus monkey, longitudinal section. ×18,500.

the microorganisms to the higher plants, with the exception of certain blue-green algae and photosynthetic bacteria in which photosensitive pigments have been found in association with dense granules. If the chemical and structural data for the plant chloroplast are taken together with similar data available for the vertebrate retinal rods, a schematic molecular model of a photoreceptor showing the pigment molecules at the interfaces between the lipid-lipoprotein and protein layers can be drawn (FIGURE 12). A smaller

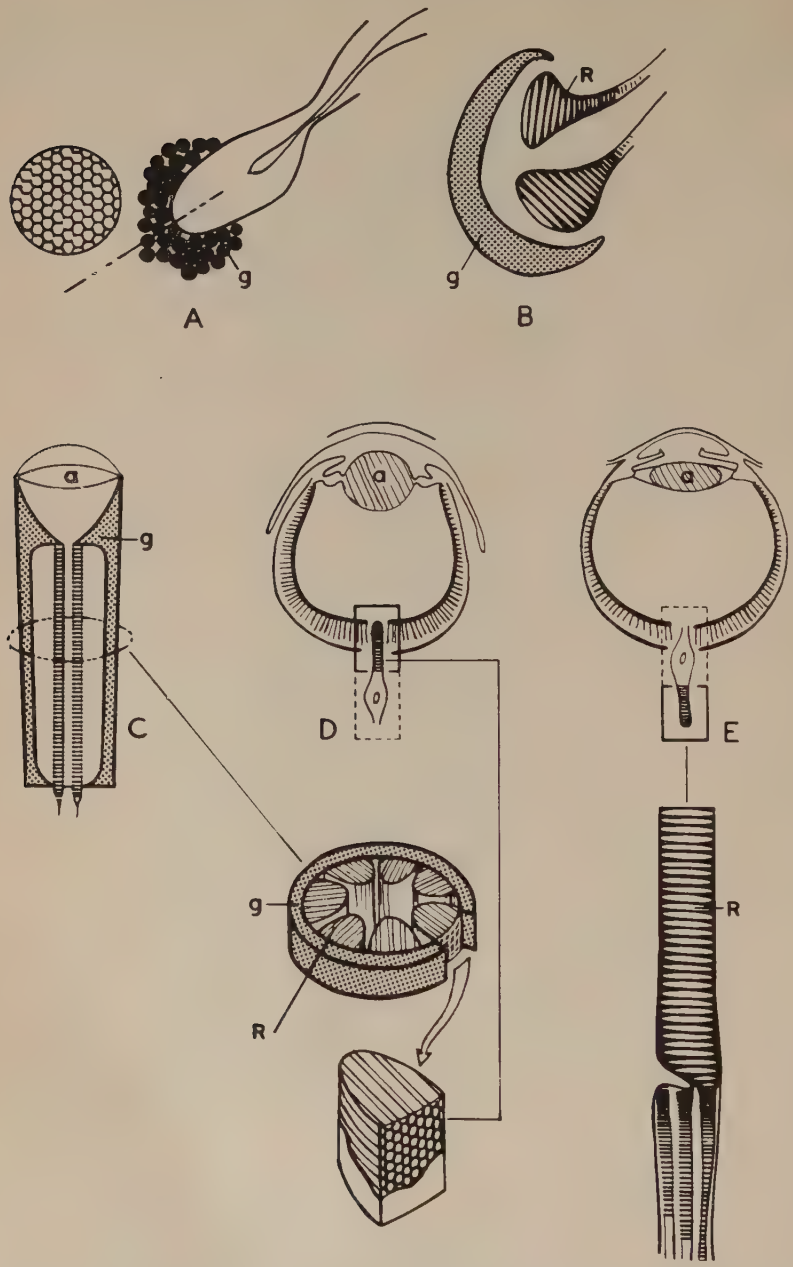

FIGURE 11. Schematic representation of a variety of eye structures: *A*, stigma (eyespot); *B*, *Planaria* eye; *C*, insect ommatidium; *D*, mollusk eye (*Octopus* and *Sepia*); and *E*, vertebrate eye. The locations of the photoreceptor elements and their microstructure are indicated: *a*, lens; *g*, pigment (sheath) granules; and *R*, retinal structures. It should be noted that in *A*, *C*, and *D* the retinal structures *R* are packed rods or tubes, and that in *B* and *E* they are packed plates or discs, which are similar to the chloroplast photoreceptors in plants.

TABLE 1
ANIMAL PHOTORECEPTORS
Average Measurements from Electron Micrographs

Animal	Photoreceptor	Diameter (μ)	Length (μ)	Total* thickness of plate or rod—Å	Number of striae per μ	Kind of packing
Invertebrates						
Protozoa						
Euglena g.	stigma (eyespot)	2.0	3	—	—	rods
Platyhelminth						
Planaria	sensory cell (retinal element)	5.0	35	140	20	plates
Arthropod						
Drosophila m.	rhabdomere (retinular cell)	1.2	60	120	23	rods
Mollusk						
Octopus v.	retinal element	1.0	—	200	20	rods
Sepia o.	retinal element	1.0	—	200	20	rods
Vertebrates						
Perch	retinal rod outer segments	1.5	40	150	60	plates
Frog	retinal rod outer segments	6.0	55	150	45	plates
Chicken	retinal rod outer segments	3.5	35	250	40	plates
Whale	retinal rod outer segments	1.4	—	200	45	plates
Cow	retinal rod outer segments	1.0	10	220	40	plates
Monkey	retinal rod outer segments	1.3	22	250	50	plates
Man	retinal rod outer segments	1.0	28	250	50	plates

* The dense edges are between 50 and 100 Å.

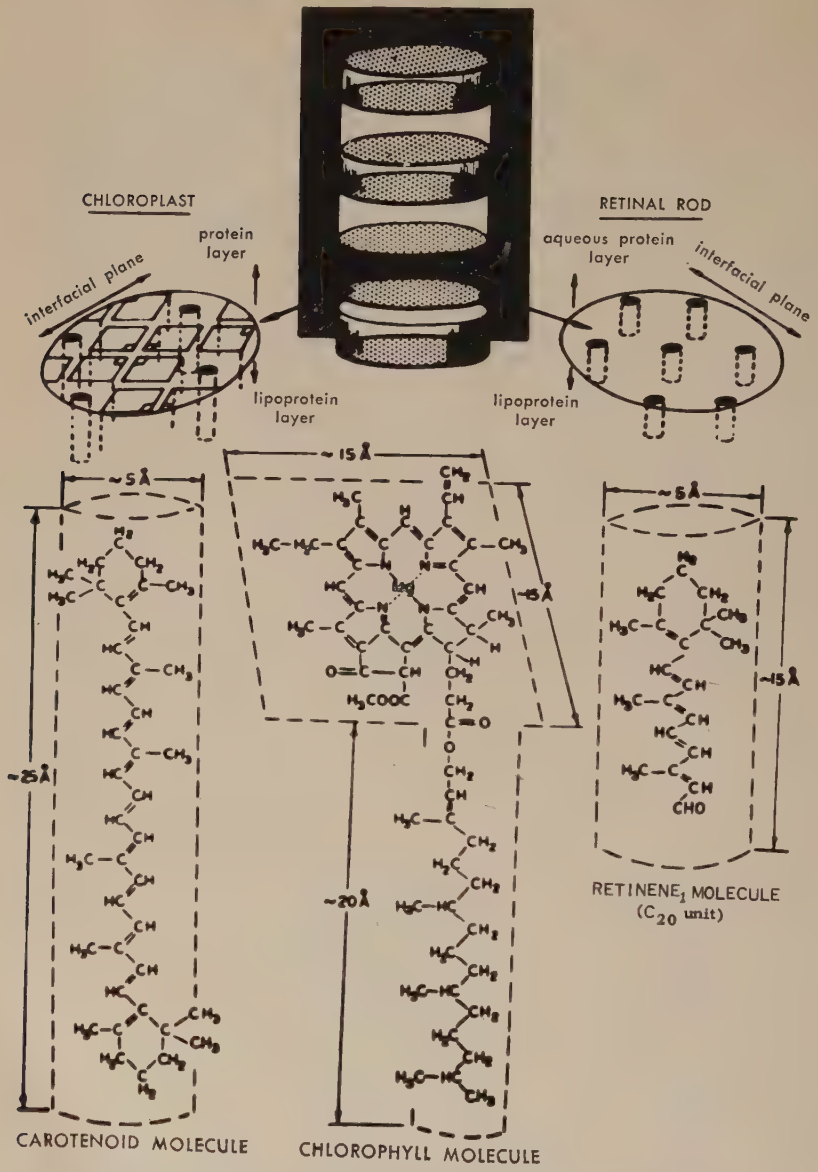

FIGURE 12. Schematic model of chloroplast and retinal rod indicating the packing of chlorophyll and carotenoids at the interfaces in the chloroplast, and the retinene in the retinal rod. This model does not indicate whether the carotenoids are in a *cis* or a *trans* configuration. Other molecular models are available.[3, 4, 18]

area of the interfaces between the protein and lipoprotein layers is greatly enlarged in the figure in order to show the molecular packing of the pigment molecules—chlorophyll and carotenoids in the chloroplast, and retinene in the retinal rod. The pigment molecules are tightly packed in the chloroplast, whereas they are loosely packed in the retinal rod.[3, 4]

Pigment-macromolecular complex. Both the photosynthetic pigment complex, chloroplastin, and the visual pigment complex, rhodopsin, can be prepared from the photoreceptors (chloroplasts and retinal rods) by extraction with 1.0 to 2.0 per cent digitonin. They appear as homogeneous complexes in the analytical ultracentrifuge, as well as by virtue of their electrophoretic patterns. The sedimentation of these extracted complexes was measured in the analytical ultracentrifuge. From the sedimentation constant, an apparent molecular weight of each of them was calculated by means of the Svedberg equation. These calculated values are similar to the molecular weights calculated on the basis of the geometry and the pigment of the photoreceptors.[4, 18, 32]

It has also been shown—from analytical data on the dry weight, the nitrogen, and the pigment concentration—that a single pigment molecule is most probably associated with each protein macromolecule.[4, 32] This was done by demonstrating that the experimentally determined pigment concentration p is substantially the same as the pigment calculated from $p = w/M'$, where w is the weight of dried residue over M', the ultracentrifuge molecular weight of the pigment-protein digitonin micelle.[4, 6] The possibility that the macromolecule may span the width of the lipoprotein layer and that there may be two pigment molecules per macromolecule cannot be excluded. As to the nature of the protein in the photosynthetic and visual complex, or how the prosthetic group is linked to the protein macromolecule, little is known at present.

Each of these photoreceptors contains pigment, lipid, lipoprotein, and water in its packed discs, plates, rods or tubes. The question remains: "How are these photoreceptor structures utilized for excitation and energy transfer?" Although this major problem has yet to be solved, some speculations have been presented. I have thought of these structures as emulsions stabilized by the pigment molecules, for, as I have observed with the electron microscope, any chemical or physical forces that affect the chemistry of the pigment molecules also disrupt the photoreceptor structure. Wald has made two suggestions for the retinal rod and cone structure: (1) that the effect of light on one molecule of pigment is to open a unimolecular hole in one of the membranes, as in a condenser, and (2) that the action of light on the complex (rhodopsin) introduces a catalysis analogous to the photographic grains of silver bromide in a photographic plate.[31, 33] It has also been suggested from resonance studies that the photoreceptors would exhibit properties of crystals, and this, together with the evidence from the electron micrographs of an ordered structure, is suggestive of an analogy to a semiconductor. Some aspects of this problem are discussed elsewhere in this monograph by Calvin. Much research has yet to be done. However, as I have already indicated, I hope we have begun to discover some of the unique structural properties of

a photoreceptor that will eventually lead us to an understanding of its function.

References

1. Wolken, J. J. & G. E. Palade. 1952. The fine structure of chloroplasts in two flagellates. Nature. **170**: 114.
2. Wolken, J. J. & G. E. Palade. 1953. An electron microscope study of two flagellates. Chloroplast structure and variation. Ann. N. Y. Acad. Sci. **56**(5): 873–889.
3. Wolken, J. J. & F. A. Schwertz. 1953. Chlorophyll monolayers in chloroplasts. J. Gen. Physiol. **37**: 111–120.
4. Wolken, J. J. 1956. Photoreceptor structures. I. Pigment monolayers and molecular weight. J. Cellular Comp. Physiol. **48**: 340–379.
5. Wolken, J. J. 1956. A molecular morphology of *Euglena gracilis*. J. Protozool. **3**: 211–221.
6. Wolken, J. J. & F. A. Schwertz. 1956. Molecular weight of algal chloroplastin. Nature. **177**: 136–138.
7. Thomas, J. B. 1955. Structure and function of the chloroplast. J. A. V. Butler & J. T. Randall, Eds. Progr. in Biophys. and Biophys. Chem. **5**: 109–139.
8. Mühlethaler, K. 1955 The structure of chloroplasts. G. H. Bourne & J. F. Danielli, Eds. Intern. Rev. Cytol. **4**: 220.
9. Granick, S. 1955. Handbuch der Pflanzenphysiologie. **1**: 507–564. Ruhland, Ed. Springer. Berlin, Germany.
10. Rabinowitch, E. I. 1956. Photosynthesis and Related Processes. II(2): 1714–1993. Interscience. New York, N. Y.
11. Schmidt, W. J. 1934. Doppelbrechung, Dichroismus und Feinbau des Aussengleides der Sehzellen vom Frosch. A. Zellforsch. u. mikroskop. Anat. **22**: 189.
12. Sidman, R. 1957. The structure and concentration of solids in photoreceptor cells studied by refractometry and interference microscopy. J. Biophys. Biochem. Cytol. **3**: 15–30.
13. Milne, L. J. & M. J. Milne. 1956. Invertebrate photoreceptor. *In* Radiation Biology. **3**: 621–692. A. Hollander, Ed. McGraw-Hill. New York, N. Y.
14. Wulff, V. J. 1956. Physiology of the compound eye. Physiol. Revs. **36**: 145–163.
15. de Vries, H. 1956 Physical aspects of the sense organs. J. A. V. Butler, Ed. Progr. in Biophys. and Biophys. Chem. **6**: 208–264.
16. Willmer, E. N. 1955. The physiology of vision. Ann. Rev. Physiol. **17**: 339–366.
17. Fauré-Fremiet, E. & C. H. Rouiller. 1957. Le flagelle interne d'une chrysomonadale: *Chromulina psammobia*. Compt. Rend. Acad. Sci. **244**: 2655–2657, and Exptl. Cell Research. **14**: 47–67 (1958).
18. Wolken, J. J. 1957. A comparative study of photoreceptors. Trans. N. Y. Acad. Sci. Ser. II. **19**: 315–327.
19. Pirenne, M. & F. H. C. Marriot. 1955. Light sensitivity of the aquatic flatworm: *Dendrocoelum lacteum*. Nature. **175**: 642.
20. Wolken, J. J., D. Mellon & G. Contis. 1957. Photoreceptor structures. II. *Drosophila melanogaster*. J. Exptl. Zool. **134**: 383–410.
21. Wolken, J. J., J. Capenos & A. M. Turano. 1957. Photoreceptor structures. III. *Drosophila melanogaster:* electron microscopy of the retinula. J. Biophys. Biochem. Cytol. **3**: 441–448.
22. Fernández-Morán, H. 1956. Fine structure of the insect retinula as revealed by electron microscopy. Nature. **177**: 742–743.
23. Goldsmith, T. H. & D. E. Philpott. 1957. The microstructure of the compound eyes of insects. J. Biophys. Biochem. Cytol. **3**: 429–438.
24 Miller, W. H. 1957. Morphology of the ommatidia of the compound eye of *Limulus*. J. Biophys. Biochem. Cytol. **3**: 421–427.
25. Wald, G. & R. Hubbard. 1957. Visual pigments of a decapod crustacean. Nature. **180**: 278–280.
26. Sjöstrand, F. S. 1949. An electron microscope study of the retinal rods of the guinea pig eye. J. Cellular Comp. Physiol. **33**: 383.

27. Sjöstrand, F. S. 1953. The ultrastructure of the outer segments of rods and cones of the eye as revealed by the electron microscope. J. Cellular Comp. Physiol. **42**: 15–44.
28. DeRobertis, E. 1956. Electron microscope observations on the submicroscopic organization of retinal rods. J. Biophys. Biochem. Cytol. **2**: 319–329.
29. Finean, J. B., F. S. Sjöstrand & E. Steinman. 1953. Submicroscopic organization of some layered lipoprotein structure. Exptl. Cell. Research. **5**: 557.
30. Goedheer, J. C. 1957. Optical properties and *in vivo* orientation of photosynthetic pigments. Thesis, University of Utrecht. Drukkerij Gebr. Janssen. Nijmegen, the Netherlands.
31. Wald, G. 1956. The biochemistry of visual excitation. Enzymes: Units of Biological Structure and Function. :355–367. O. H. Gaebler, Ed. Academic Press. New York, N. Y.
32. Hubbard, R. 1954. The molecular weight of rhodopsin and the nature of rhodopsin-digitonin complex. J. Gen. Physiol. **37**: 381–398.
33. Wald, G. 1954. On the mechanism of the visual threshold and visual adaptation. Science. **119**: 887.

HISTOCHEMICAL STUDIES ON PHOTORECEPTOR CELLS

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Two topics concerning the structure and chemistry of the retinal pigment epithelium and the adjacent photoreceptor cells are presented in this paper. The first of these is a histological study of the zone that connects the pigment epithelium and the photoreceptors. The second is a consideration of some spectral absorption curves of intact rod outer segments.

The Interstitial Zone of the Rodent's Retina

Feder and I have modified the freeze-substitution method of histological fixation.[1] The technique preserves structure and conserves certain chemical substances better than more common methods of fixation. In brief, intact small eyes—up to the size of the mature guinea pig's eye—or posterior halves of large eyes are frozen rapidly at −175° C. The ice is then dissolved or "substituted" in a cold solvent containing fixatives. Most of the specimens were substituted at −70° C. in 5 per cent mercuric chloride in ethanol or 1 per cent osmic acid in acetone. After substitution the eyes were embedded, sectioned, and stained in the usual ways.

FIGURE 1 shows a section of the retina of an albino mouse stained by Cason's variant of the Mallory method.[2] The vitreal surface is at the bottom. In the albino mouse the pigment cells *p* at the upper border of the retina lack pigment. A blue-staining interstitial area *i* lies between the pigment cells and the rod outer segments *os*. The rod inner segments *is* have an accumulation of closely packed mitochondria in their distal, or ellipsoid, portions and ribonucleoprotein in their proximal, or myoid, portions.[3] All the enzymes of photoreceptor cells so far localized are found in the inner segments, none in the photoreceptive outer segments.[4, 5] Below the photoreceptor cell nuclei is a series of small red-stained spheres *s* representing parts of the well-preserved synaptic apparatus between photoreceptor and bipolar cells. Retinal capillaries *c* penetrate as far as this synaptic zone, while the chorioidal circulation reaches only to Bruch's membrane, at the base of the pigment cells. The tissue between is avascular.

The albino mouse's eye shown in FIGURE 2 was substituted and fixed at −70° C. in osmic acid in acetone, and an unstained 5 μ section was examined with phase contrast optics. Three components are seen in the interstitial zone:

(1) The apical cytoplasm of the pigment cell contains granules *g* of various sizes from the limit of resolution of the light microscope up to about 1.5 μ.

(2) Thin, irregularly shaped fibrils *f* are seen, apparently issuing from the distal border of the pigment cell. In some instances the fibrils appear to be in contact with the granules. The fibrils often point directly to the tips of

the rods, but at times extend a considerable distance between the rod outer segments.

(3) A homogeneous material of low contrast *m* appears to occupy most of the space between pigment cells and rod outer segments not occupied by the fibrils. This last material is called the interstitial matrix.

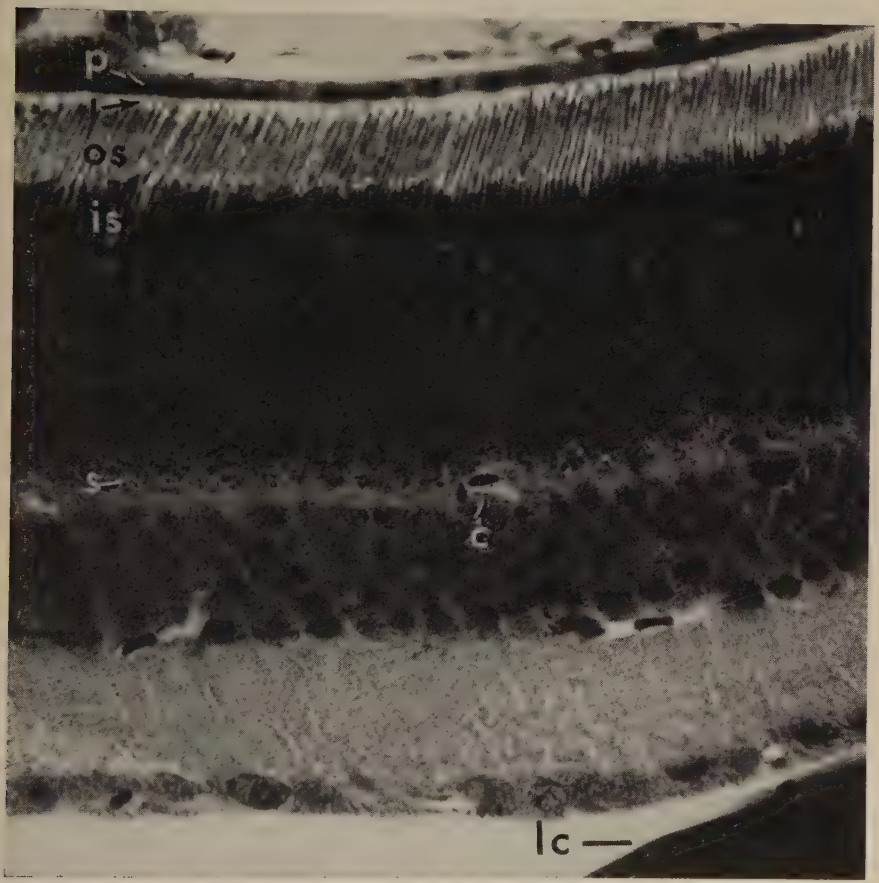

FIGURE 1. Retina of the albino mouse showing: *p*, pigment epithelium; *i*, interstitial zone; *os*, rod outer segments; *is*, rod inner segments; *s*, rod-bipolar cell synapse; *c*, capillary; and *lc*, lens capsule. The retinal section was freeze-substituted in 5 per cent $HgCl_2$ in ethanol, embedded in paraffin, sectioned at 5 μ and stained with Cason's rapid variant of Mallory's stain.[2] ×735.

With the periodic acid-Schiff (PAS) stain (FIGURE 3), both the intracellular granules and the fibrillar fronds of the pigment epithelial cells are colored red, as are the rod outer segments. These red-stained structures probably fall into the loosely defined class of complex carbohydrates called glycoproteins. However, further staining reactions show that these PAS-positive structures may not be composed of the same complex carbohydrates, although

all are stained similarly here. In this preparation (FIGURE 3) the homogeneous interstitial matrix is unstained and is represented by the empty spaces between the red fibrils.

FIGURE 4a illustrates another specimen stained by the PAS method. For comparison, the specimen in FIGURE 4b was stained both by the periodic acid-Schiff method and by alcian blue, a dye that imparts a clear blue color to acid mucopolysaccharides. The granules within the pigment cell stain red, as before (FIGURES 3 and 4a), but the fibrils apparently take both dyes

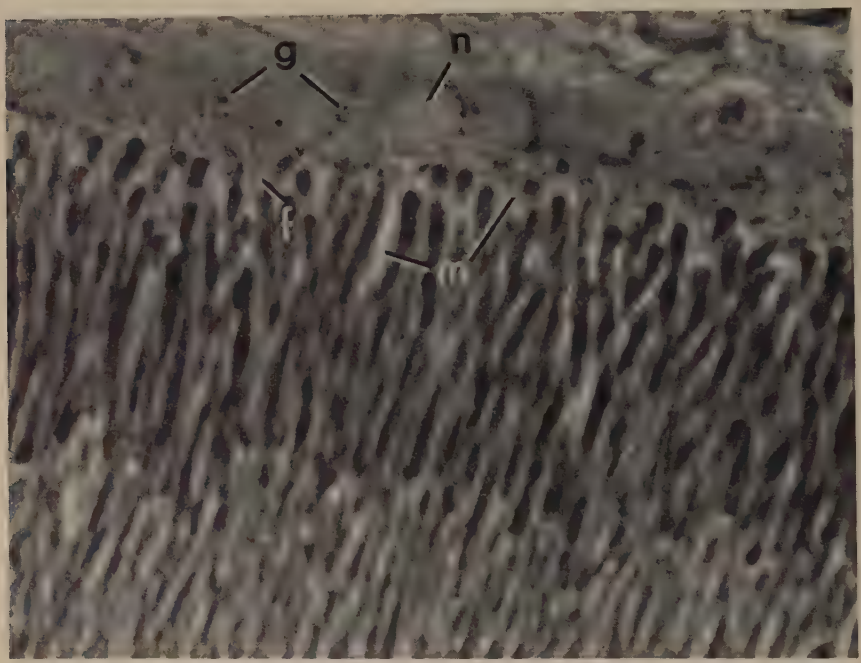

FIGURE 2. Retinal interstitial zone of the albino mouse showing: *g*, granules; *f*, fibrils; *n*, nucleus of pigment epithelial cell; and *m*, interstitial matrix. The section was freeze-substituted in 1 per cent osmic acid in acetone, embedded in paraffin, sectioned at 5 μ, unstained, and examined with phase-contrast optics. ×1800.

and appear purple (FIGURE 4b). The homogeneous matrix extending between and beyond the fibrils has the clear blue color characteristic of acid mucopolysaccharides (FIGURE 4b).

The interstitial matrix stains metachromatically with toluidine blue or azure A (FIGURE 5) even after the specimen is dehydrated with ethanol. The fact that the reddish metachromasia persists after dehydration also indicates the presence of acid mucopolysaccharides. The acid mucopolysaccharide appears to be in the interstitial matrix *m* only, of the three components under consideration. The unstained zone *f* immediately at the apical border of the pigment cells indicates that the granules and fibrils contain chemical substances other than acid mucopolysaccharides.

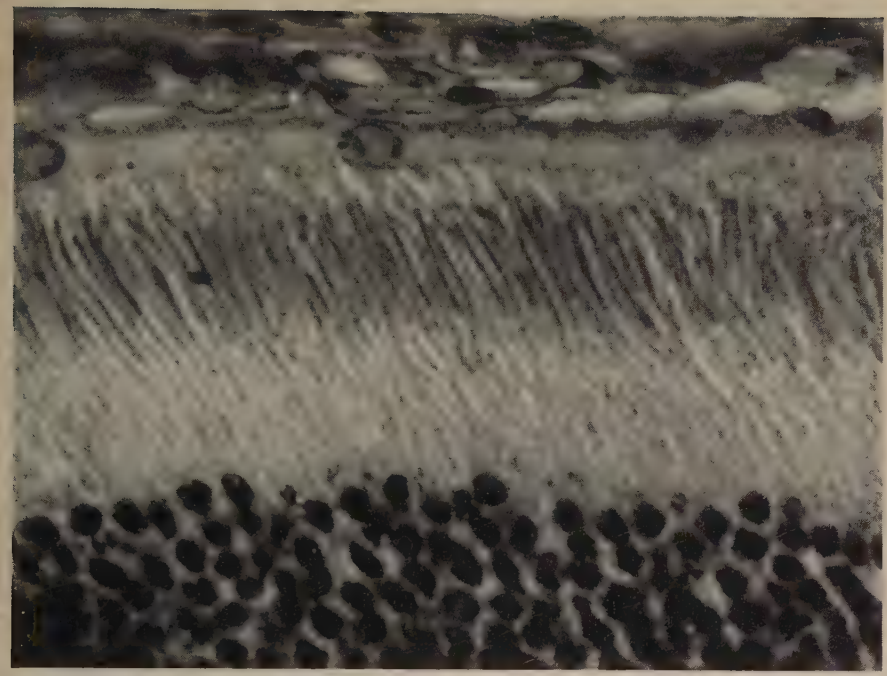

FIGURE 3. Retina of the albino mouse processed as in FIGURE 1 and stained with periodic acid-Schiff and Harris's hematoxylin. ×1360.

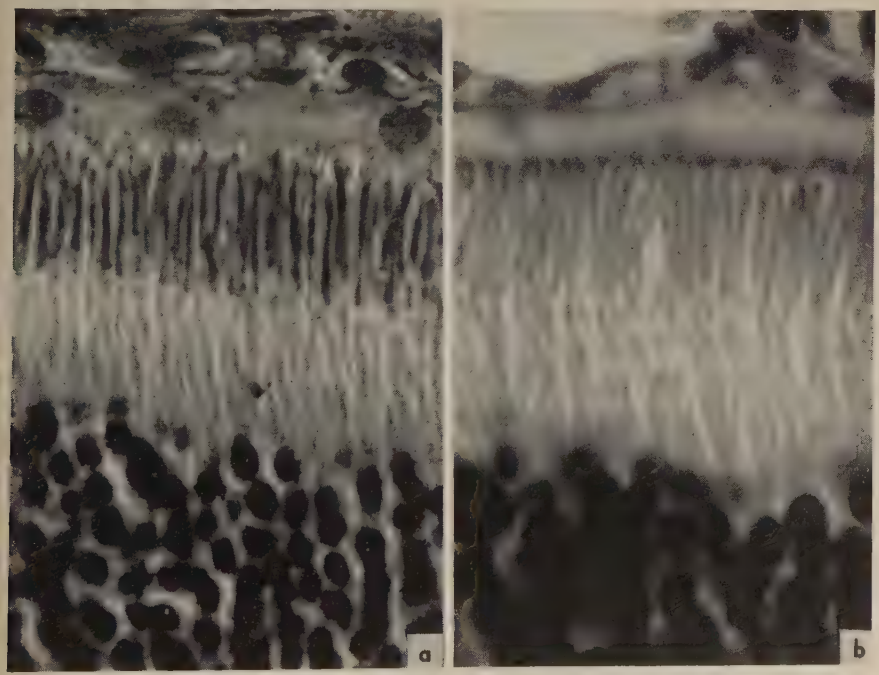

FIGURE 4. Retina of the albino mouse (a) processed as in FIGURE 3 and (b) processed similarly, but stained with alcian blue and periodic acid-Schiff. ×1280.

In another section stained with the combined alcian blue and PAS methods (FIGURE 6) the extent and density of the fibrillar material *f* can be better appreciated. In an oblique view (FIGURE 7) many rods are cut tangentially and transversely. The granules *g* and fibrils *f* show clearly, and a clear blue sheath can be seen surrounding these rods. This interstitial mucopolysaccharide matrix *m* can be traced for a considerable distance along the length of the rod outer segments.

In summary, the granules in the pigment epithelial cells are PAS-positive and, like the rod outer segments, remain red when the combined alcian blue-

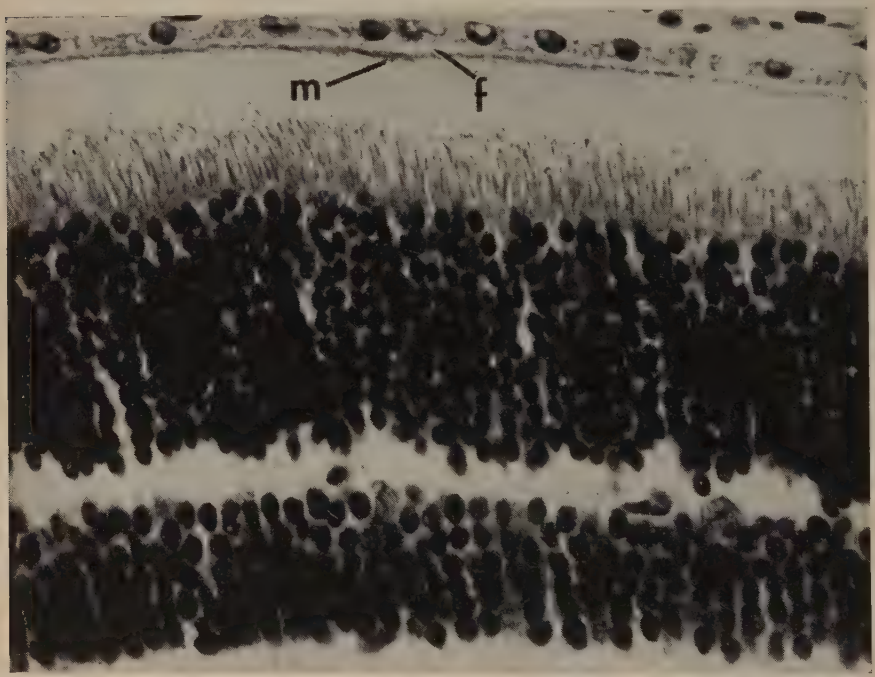

FIGURE 5. Retina of the albino mouse showing: *m*, interstitial matrix and *f*, fibrils. The section was processed as in FIGURE 1, stained with 0.1 per cent azure A in 30 per cent ethanol, and dehydrated in graded ethanols. ×725.

PAS stain is applied. The fibrils extending from the pigment cells either accept both dyes or, which is equally likely, they consist of a PAS-positive substance to which an alcian blue-positive substance is closely applied. The homogeneous matrix is not colored by the PAS stain and is demonstrated to contain an acid mucopolysaccharide by its metachromatic reaction with toluidine blue or azure A and its clear blue color after staining with alcian blue. However, even the metachromasia and the alcian blue stain may not represent the same chemical substance, for the latter is strong and the former is absent in many of our specimens.

These characteristics of the pigment cell and interstitial zone have been recognized only imperfectly in the past because satisfactory fixation was

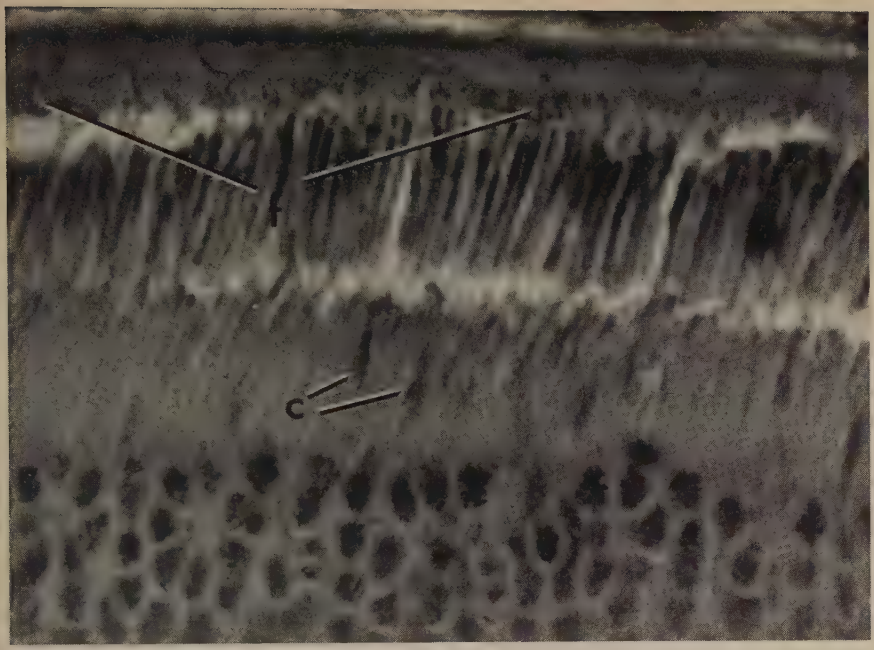

FIGURE 6. Retina of the albino mouse showing: *c*, ellipsoids of cone cells and *f*, fibrils. The section was freeze-substituted in 5 per cent $HgCl_2$ in ethanol, embedded in paraffin, sectioned at 5 μ, and stained with alcian blue and periodic acid-Schiff. ×1015.

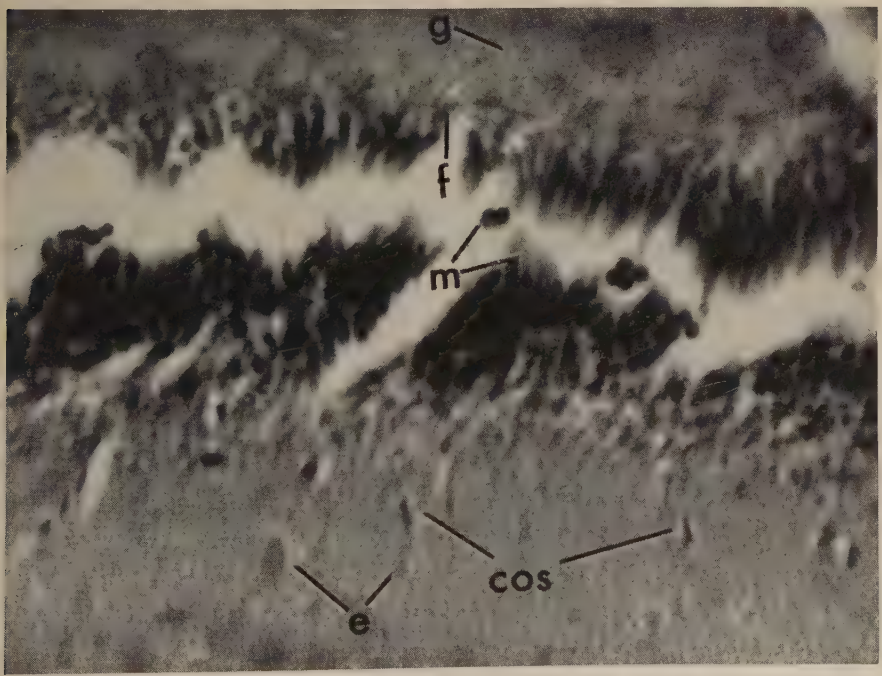

FIGURE 7. Retina of the albino mouse showing: *g*, granules; *f*, fibrils; *m*, interstitial matrix; *cos*, cone outer segments; and *e*, cone ellipsoids. Section was processed as in FIGURE 6. ×1585.

difficult to achieve. Even before the cellular arrangements in the vertebrate retina had been adequately described, Henle[6] and others had suggested that the rods were embedded in a homogeneous matrix. However, little evidence of such a matrix was obtained, either in teased or in sectioned and stained retinas, and Henle's idea lost favor.[7] In 1909 Kolmer[8] presented some evidence for a secretory function of the pigment epithelium, but he withdrew the claim at a later date.[9] Six years ago Lillie,[10] using a form of freeze-substitution fixation, described metachromasia of rod outer segments. Subsequently the metachromasia was localized to a fibrillar and amorphous

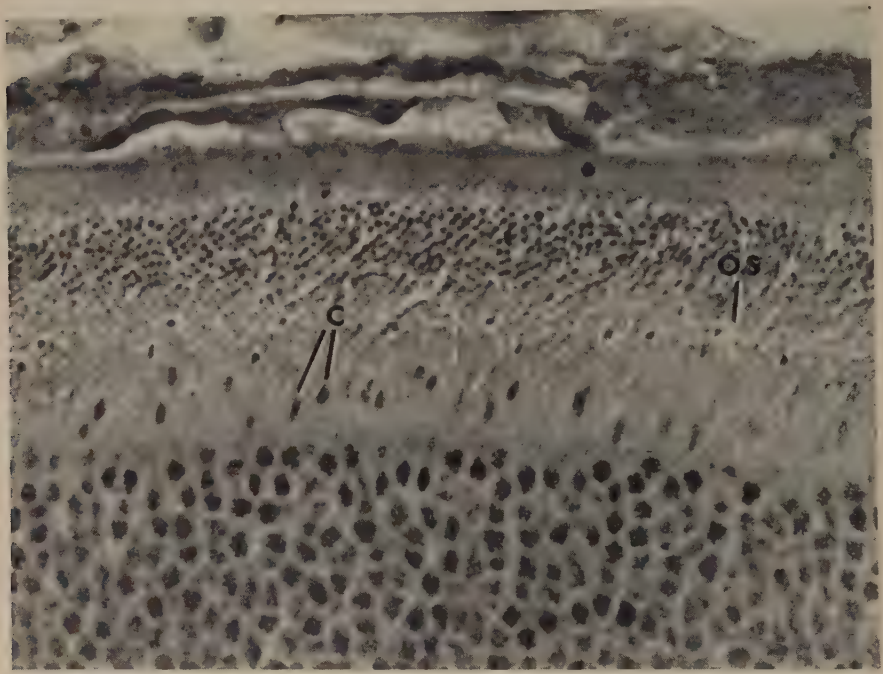

FIGURE 8. Retina of the albino mouse showing: *c*, cones and *os*, outer rod segments. Processed as in FIGURE 6. ×790.

material between the rod outer segments,[4] but preservation was too incomplete and irregular for detailed study. Later the homogeneous acid mucopolysaccharide component was demonstrated with alcian blue in the human retina,[11, 12] but apparently the fixatives used did not permit demonstration of its metachromasia. So it is that we have now come full circle and again believe that an interstitial matrix material does exist. Furthermore, the pigment epithelial cells with their granules and fibrils have the appearance of secretory cells, supporting Kolmer's opinion of 1909.

The significance of the interstitial substances is not clear. FIGURE 9, the retina of a pigmented rat, shows that the homogeneous interstitial matrix *m*, which is colored by alcian blue, extends well beyond the pigment granules. The interstitial matrix, therefore, is not directly related to the "pigment-free

pigment granules" in the albino retinas illustrated in FIGURES 1 through 8. The matrix is probably extracellular; it extends far down between the photoreceptor cells, and in the pigeon and the monkey it even reaches between the inner segments. On the other hand, we do not yet know precisely how far the projections of the pigment cells extend between the rod outer segments in mammals and, as a result, we cannot say whether the fibrillar component is intra- or extracellular. Observations with the electron microscope should provide the answer.

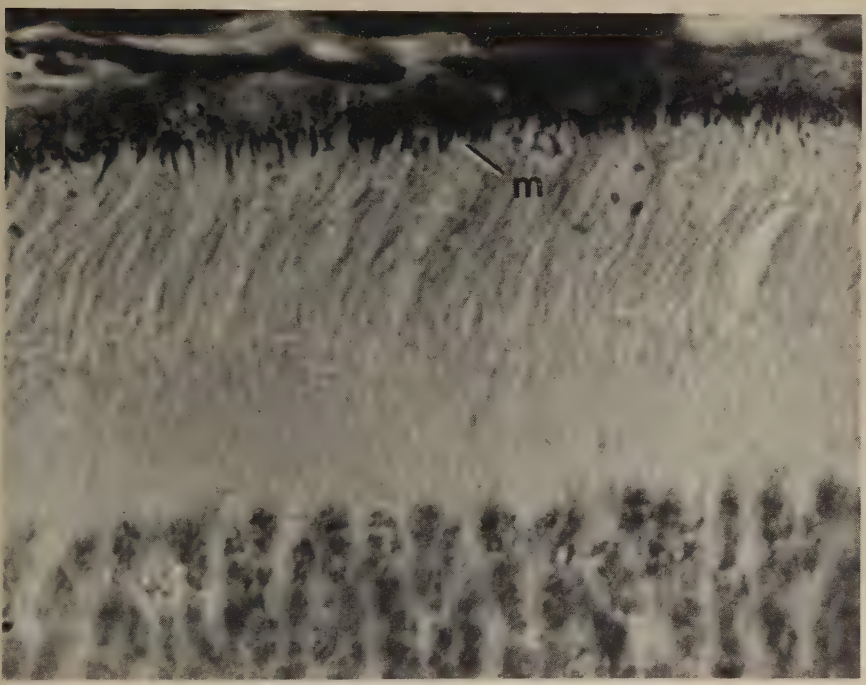

FIGURE 9. Retina of the black rat showing *m*, the interstitial matrix. Processed as in FIGURE 6. ×1440.

There is considerable variation in the number and the disposition of the PAS-positive granules of the pigment cells as well as in intensity and distribution of metachromasia in the interstitial zone. These variations exist even within inbred strains of mice, and as yet we have been unable to correlate the variability with strain, age, sex, or state of light adaptation.

However, even in the absence of more specific information on its physiology, the anatomy at once suggests two important functional considerations:

(1) Since the photoreceptor zone is avascular, any metabolites, such as vitamin A, which interchange between photoreceptor cells and the blood in the chorioidal capillaries must cross the interstitial zone. Metabolites might be altered chemically or their rate of movement controlled in the interstitial zone.

(2) The refractive index of the homogeneous interstitial material may be of some importance. The efficiency with which outer segments concentrate light depends on the shape and size of the segment and on the ratio of refractive indexes between the outer segment and its environment. For example, O'Brien[13] showed in a model system that a ratio of refractive indexes of 1.022 for a cone outer segment tapering at an angle of 8° would account for the Stiles-Crawford effect (that cones are more sensitive to axial than to oblique rays of light). Rod and cone outer segments were found subsequently to have refractive indexes averaging 1.41 and 1.385, respectively.[14] The approximation was made[14, 15] that the medium surrounding the outer segments has a refractive index comparable to that of serum, and a ratio was obtained supporting O'Brien's hypothesis. If the interstitial matrix containing acid mucopolysaccharide, which we now know encloses the outer segments, has a higher refractive index than that of serum, the ratio of refractive indexes would be even closer to O'Brien's figure. An accurate measure of the refractive index of the interstitial substance may be of great value for an understanding of the functional importance of the retinal interstitial zone.

Two further facts about the photoreceptive zone of the retina are worth recording. In mouse retinas we regularly find cells interposed between the pigment epithelium and the rods (FIGURE 10). These cells have round or oval nuclei located eccentrically, and a relatively large volume of cytoplasm. The nuclei stain darker than those of the pigment epithelial cells. Often the cell has a broad base abutting the apical border of the pigment epithelium (FIGURE 10b). The most striking attribute of these "interstitial cells" is their complement of uniformly large and round, strongly PAS-positive granules, which fill the cytoplasm (FIGURE 10b). The granules are not metachromatic when stained with azure A or toluidine blue (FIGURE 10a), thus making these cells easily distinguishable from the mast cells common in the chorioid. The granules, unlike rod outer segments, do not stain for lipids with sudan black B in paraffin-embedded tissues; neither do they stain prominently with eosin. The interstitial cells apparently wander from a position near the pigment epithelium toward the rod inner segments (FIGURE 10a) and, characteristically, they appear near the myoids of the inner segments as isolated nuclei with cytoplasmic fragments attached, or as even more severely fragmented detritus. An average of between two and four interstitial cells is seen in each 5 μ section of mouse retina. The significance of these cells is unknown.

By way of digression, it should be said that the zone of the rod inner segments contains a number of plump ovoid structures (FIGURE 6, *c*; FIGURE 7, *e*); these are the ellipsoids of cone cells and, in a few places, their PAS-positive outer segments can be discerned (FIGURE 7, *cos*). The presence of significant numbers of cones in rodent retinas, particularly in the mouse and the rat, has generally been denied, but they are clearly seen in freeze-substituted retinas stained by any of several methods. FIGURE 8 gives a better demonstration of their frequency in the mouse. The ratio of cones to rods is about one-tenth in the mouse, one-tenth to one-twentieth in the rat, and

one-fifth in the guinea pig. The rodent cones are quite similar to those of other mammals in both appearance and staining properties.

Light Absorption by Rhodopsin in Intact Rods

Suspensions of rod outer segments in ordinary aqueous media are too turbid for adequate spectrophotometric study. However, rods are remarkably uniform in refractive index and concentration of solids.[14] When isolated rod outer segments are suspended in 40 per cent bovine plasma albumin adjusted to isotonicity, the refractive indexes of rods and medium

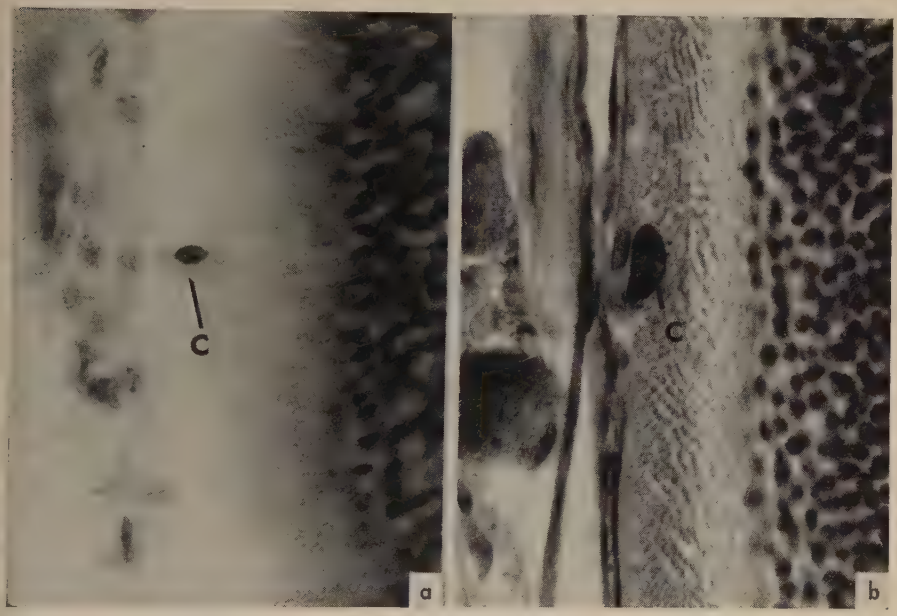

FIGURE 10. Retina of the albino mouse showing: (a) *c*, interstitial cell processed as in FIGURE 6, stained in 0.01 per cent toluidine blue in H_2O, dehydrated in graded ethanols; and (b) *c*, interstitial cell, processed as in FIGURE 6. ×860.

match sufficiently to reduce scatter and allow direct spectrophotometry of the clarified suspension.[16]

The absorption spectrum of such a suspension of dark-adapted frog rods is shown in FIGURE 11. The rods absorb maximally at about 508 mμ. After bleaching with white light the absorption above 460 mμ falls and that below 460 mμ rises. The acid difference spectrum has a maximum at 515 mμ. The position of the peak at 508 mμ on the direct spectrum may be distorted by blue-absorbing impurities, and the true peak may be at a wave length longer than 508 mμ.

These values for the absorption maximum of frog rods differ significantly from the absorption maximum at 502 mμ of frog rhodopsin extracted with digitonin. Others have obtained a similar shift toward the red in the position of the peak on the difference spectrum,[17-19] and several possible explana-

tions for the shift have been advanced.[19] However, some explanations that account for altered difference spectra would not be applicable to the shifted direct spectrum obtained in albumin (FIGURE 11). The most plausible explanations of the shifted direct spectrum are: (1) a second pigment that absorbs at longer wave lengths is present; (2) the albumin medium or some other methodological artifact causes a displacement of the absorption curve in some way unrelated to the properties of the light-sensitive pigment; or (3) the light-sensitive pigment is different in the intact cells and in solution.

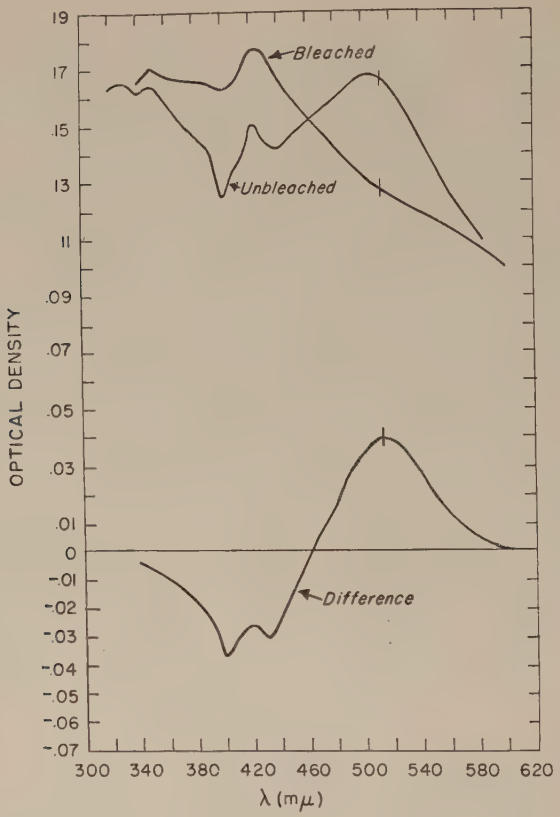

FIGURE 11. Frog rods in 40 per cent albumin, *p*H 5.5.

No evidence for a second pigment in these preparations has been obtained by partial bleaching, although the data are not adequate to rule out the possibility that a second pigment might absorb maximally at a wave length fairly close to the peak of rhodopsin, at perhaps 520 mμ. The albumin medium is not responsible for the shift because similar, although flatter, curves are obtained from rods suspended in sucrose and measured with a ground glass interposed in the light path (FIGURE 12), using modifications of Shibata's technique[20] suggested by Charles Greenblatt of the National Institutes of

Health (personal communication). Feder and I have obtained similar data on retinal smears and spreads mounted in the spectrophotometer in a number of ways. A shift of comparable magnitude in the position of maximal absorption is found in intact cattle and monkey rods; the effect is not limited to the frog. Finally, the digitonin commonly used to extract rhodopsin is not responsible in any specific way for pulling the absorption curve toward the short wave lengths for, when rods are disrupted with ultrasonic vibrations, both the direct absorption maximum and the peak on the difference spectrum are near the digitonin value at 502 mμ (FIGURE 13). Thus the shifted spectrum seems to imply a difference between the states of rhodopsin in

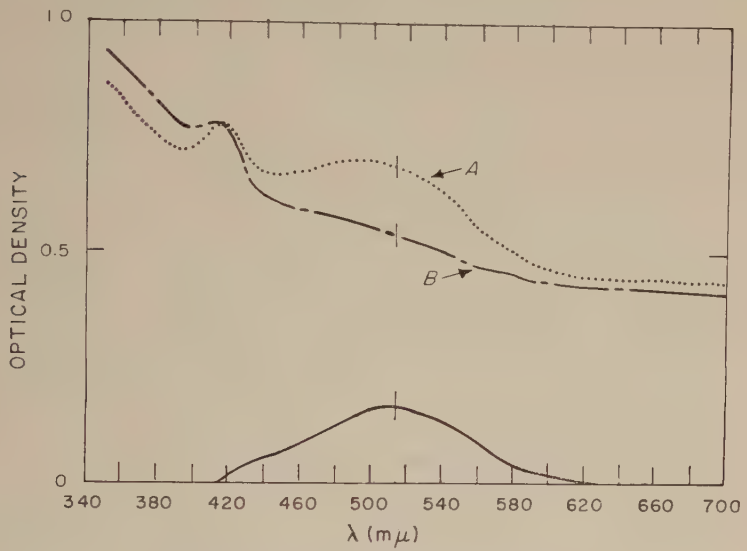

FIGURE 12. Frog rods in 40 per cent sucrose.

intact rods and of rhodopsin dispersed in small fragments of rods or in solution. A second, less likely alternative is that the shift results from the summation of the absorption curves of rhodopsin and of a second pigment absorbing only about 20 mμ from rhodopsin, toward the red.

Two important publications on this topic have recently appeared. Denton[21] has described a very effective method for obtaining absorption spectra on intact excised retinas. His absorption curves, which have a far more favorable ratio of maximal to minimal absorption than ours, show a comparable shift of the absorption maximum toward the red.

Wald and Brown[22] have measured the absorption characteristics of human rhodopsin. The extracted pigment absorbs maximally at 493 mμ. However, rhodopsin in isolated human retinal rods absorbs maximally at 500 mμ, and the curve corresponds in form and position to the spectral sensitivity of human rod vision. Thus, agreement is general that the absorption spectrum

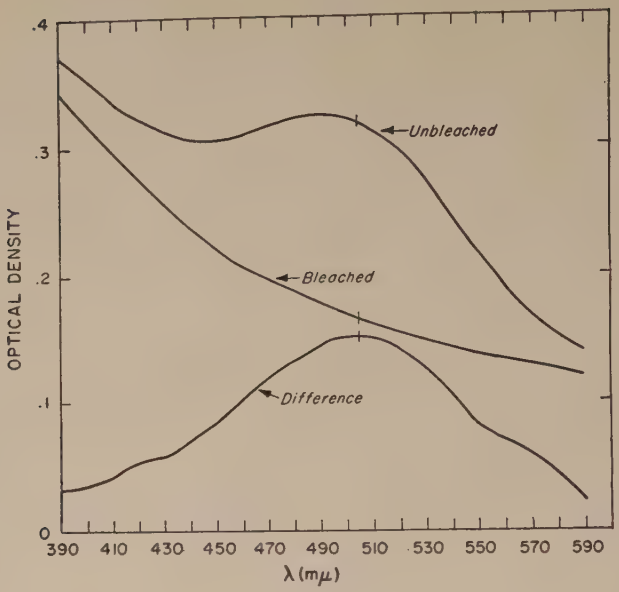

FIGURE 13. Ultrasonic disruption of frog rods.

of rhodopsin in rods lies at higher wave lengths than the spectrum of extracted rhodopsin, and that the spectral sensitivity of human rod vision relates to the absorption of rhodopsin *in situ*.

Summary

Some properties of the retinal interstitial zone, which lies between the pigment epithelium and the rod outer segments, have been described. Granules containing glycoprotein are found in the apexes of the pigment epithelial cells. Fibrils of similar chemical nature form a part of the processes that make up the apical border of the pigment cells, or perhaps actually project beyond the pigment cells toward the rod and cone outer segments. A homogeneous interstitial matrix containing acid mucopolysaccharide fills the space between the pigment cells and the photoreceptor outer segments, and also lies between the outer segments themselves. This interstitial zone has become much more accessible for study since the introduction of a modified freeze-substitution method of histological fixation; its strategic anatomical location with reference to the photoreceptive parts of rods and cones would seem to justify renewed attention.

Just as the study of some morphological and chemical properties of the interstitial zone proved feasible in well-preserved retinas, so the study of photosensitive pigments *in situ* yielded information not obtained by study after they had been extracted from the retina. The absorption maximum of rhodopsin in intact rods is shifted toward the longer wave lengths compared to the maximum of extracted rhodopsin.

References

1. Feder, N. & R. L. Sidman. 1958. Methods and principles of fixation by freeze-substitution. J. Biophys. Biochem. Cytol. **4**: In press.
2. Lillie, R. D. 1954. Histopathologic Technic and Practical Histochemistry. 2nd ed. 501 pages. Blakiston. New York, N. Y.
3. Sidman, R. L. & G. B. Wislocki. 1954. Histochemical observations on rods and cones in retinas of vertebrates. J. Histochem. Cytochem. **2**: 413–433.
4. Wislocki, G. B. & R. L. Sidman. 1954. Chemical morphology of the retina. J. Comp. Neurol. **101**: 53–100.
5. Lowry, O. H., N. R. Roberts & C. Lewis. 1956. The quantitative histochemistry of the retina. J. Biol. Chem. **220**: 879–892.
6. Henle, J. 1855–1873. Retina. *In* Handbuch der systematischen Anatomie des Menschen. **2**: 636–670. Vieweg. Braunschweig, Germany.
7. Schultze, M. 1873. The retina. *In* Manual of Human and Comparative Histology. **3**: 218–298. S. Stricker, Ed. New Sydenham Soc. London, England.
8. Kolmer, W. 1909. Über einen sekretartigen Bestandteil der Stäbchenzapfenschicht der Wirbeltierretina. Arch. ges. Physiol. Pflüger's. **129**: 35–45.
9. Kolmer, W. 1925. Bemerkungen über Adaptationsvorgänge in den Sehelementen. Arch. Ophthalmol. Graefe's. **115**: 310–313.
10. Lillie, R. D. 1952. Histochemical studies on the retina. Anat. Record. **112**: 477–495.
11. Mazanec, K. & Z. Pliczka. 1955. Mikroskopicka histochemie svetlocivych elementu sitnice. Československ. oftalmol. **11**: 227–234.
12. Zimmerman, I. 1958. Some applications of special staining methods for the demonstration of acid mucopolysaccharides to ophthalmic histology and pathology. Trans. Am. Acad. Ophthalmol. Otolaryngol. In press.
13. O'Brien, B. 1951. Vision and resolution in the central retina. J. Opt. Soc. Am. **41**: 882–894.
14. Sidman, R. L. 1957. The structure and concentration of solids in photoreceptor cells studied by refractometry and interference microscopy. J. Biophys. Biochem. Cytol. **3**: 15–30.
15. Barer, R. 1957. Refractometry and interferometry of living cells. J. Opt. Soc. Am. **47**: 545–556.
16. Barer, R. & R. L. Sidman. 1955. The absorption spectrum of rhodopsin in solution and in intact rods. J. Physiol. **129**: 60–61.
17. Arden, G. B. 1954. Light-sensitive pigment in the visual cells of the frog. J. Physiol. **123**: 377–385.
18. Dobrowolski, J. A., B. K. Johnson & K. Tansley. 1955. The spectral absorption of the photopigment of *Xenopus laevis* measured in single rods. J. Physiol. **130**: 533–542.
19. Rushton, W. A. H. 1956. The difference spectrum and the photosensitivity of rhodopsin in the living human eye. J. Physiol. **134**: 11–29.
20. Shibata, K., A. A. Benson & M. Calvin. 1954. The absorption spectra of suspensions of living micro-organisms. Biochim. Biophys. Acta **15**: 461–470.
21. Denton, E. 1958. The visual pigment of the conger eel. Proc. Roy. Soc. London. **B148**: 257–269.
22. Wald, G. & P. K. Brown. 1958. Human rhodopsin. Science. **127**: 222–226.

OPTICS OF THE INSECT EYE

By Hessel de Vries and Jan W. Kuiper

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Some years ago von Frisch[1] discovered that the bee could detect the plane of polarization of light scattered by the blue sky. By this means it is possible to reconstruct the position of the sun if an appropriate computing system is available. This, finally, is important for orientation purposes, in case the sun is covered by clouds. It is obvious that this ability to detect polarized light introduces two different problems: how is the plane of polarization detected in different parts of the visual field, and how is the information obtained integrated by the nervous system. In the course of our experimental work, a third problem presented itself, namely, that the current ideas about image formation and the visual field of individual ommatidia required correction. This last point has continued to be of interest, in spite of the fact that Baylor and Smith have demonstrated that the plane of polarization is not detected by the eye alone.* The primary phenomenon has turned out to be the reflection pattern of light on the environment, which depends on the plane of polarization. In this way the detection of the plane of polarization is reduced to brightness discrimination. No evidence for a "direct" detection of the plane of polarization could be found.

Detection of Polarized Light

In 1844 Haidinger discovered that the naked human eye can detect the polarization of light by means of what are now called the Haidinger brushes. When looking at a white field through a polaroid filter, one observes a yellow shape that can be described as a yellow figure eight. It is seen in the position of the number 8 if the plane of polarization, that is, the plane containing the electric vector, is horizontal. Helmholtz explained this phenomenon by assuming that the molecules in the yellow macular pigment are oriented with radial symmetry, that is, that they are perpendicular to the fibers radiating outward from the fovea. It would follow from this that the quadrants of the fovea behind the pigment that is oriented parallel to the plane of polarization receive less light than do the other two quadrants. Consequently, the eye perceives a vaguely defined figure eight. Since the pigment absorbs blue light, this figure is dark when the incident light is blue, whereas it appears yellow when the incident light is white. This is an entoptic phenomenon and, for that reason, it is not only difficult to study, but sometimes even difficult to observe. Some years ago, however, de Vries *et al.*[2, 3] developed a more objective method for studying it. From our measurements we were able to conclude that the degree of orientation of the pigment was small—only about 5 per cent. This is not unreasonable, since nature has not

* E. R. Baylor, and F. E. Smith, in a paper presented at a symposium on pattern recognition, Ann Arbor, Mich., October, 1957 (to be published); also in a paper presented at a conference on biophysics, Cambridge, Mass., February, 1958 (to be published).

oriented these molecules on purpose—the effect is of no use. We also studied the magnitude of the dichroic effect as a function of wave length and found that it agreed well with the spectral absorption curve of the yellow pigment (FIGURE 1).

When it was still assumed that the insect eye could detect the plane of polarization, it was obvious that this detection was a far more fundamental phenomenon than is the Haidinger effect in human vision. It has long been known that the insect's ability to detect the plane of polarization is not due to the presence of "polaroid" filters, either in the lenses or elsewhere in the optical path.[4] When the insect eye is observed through a rotating polaroid,

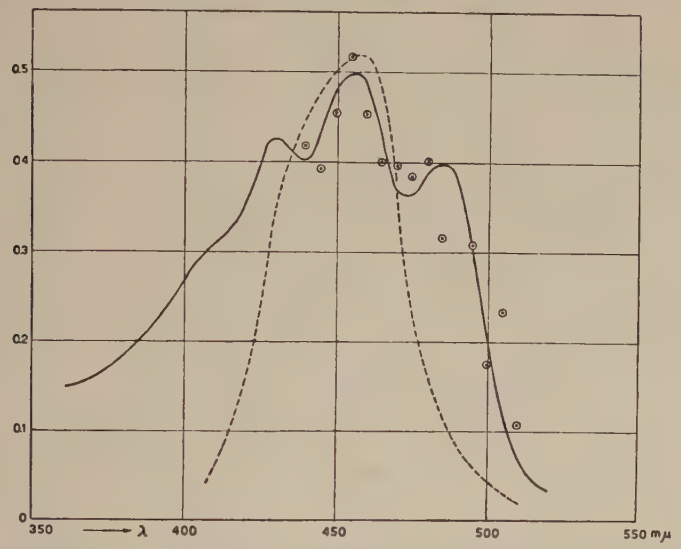

FIGURE 1. The absorption curve of yellow pigment in the fovea, represented by the solid line. The dots show the magnitude of dichroic effect in foveal pigment, and fit very well on the absorption curve of the yellow pigment. The dotted line, which is the sensitivity curve of blue receptors, is presented for comparison.

no obvious changes in intensity of the transmitted light are found and, if the human eye cannot detect any difference of intensity, it is improbable that any dichroic effect of importance to the insect is present.

Before going further, a brief description of the insect eye is in order. Called a compound eye, it can be described as a half sphere, although it is actually shaped more irregularly than that. Its surface is subdivided into small lenses each having an average diameter of about 30 μ. The optimal size of the lenses is discussed elsewhere.[5] In the focal plane of this lens the sense cells begin; their length is about 80 μ. In each ommatidium there are seven cells surrounded by a dark pigment. The cells are subdivided into a part with a low refractive index—about 1.33—and a thin thread, the rhabdomere, with a refractive index of about 1.5. The arrangement of the cells or, more especially, the rhabdomeres, is not always the same. In the eye of

Hymenoptera—for example, that of the bee—the cells are arranged in a single cylinder in such a way that the rhabdomeres form one central rhabdom. In the eye of Diptera—for example, that of the blowfly—the rhabdomeres are arranged in the typical pattern shown in FIGURE 2.

In experiments to determine the effects of polarized light, which are also discussed by Menzer and Stockhammer,[6] who provided the next step in our understanding of this phenomenon, the half-spherical eye was cut from the head along a plane parallel to its base. The position of this cut was so chosen that the cut went through the sense cells. This preparation was then

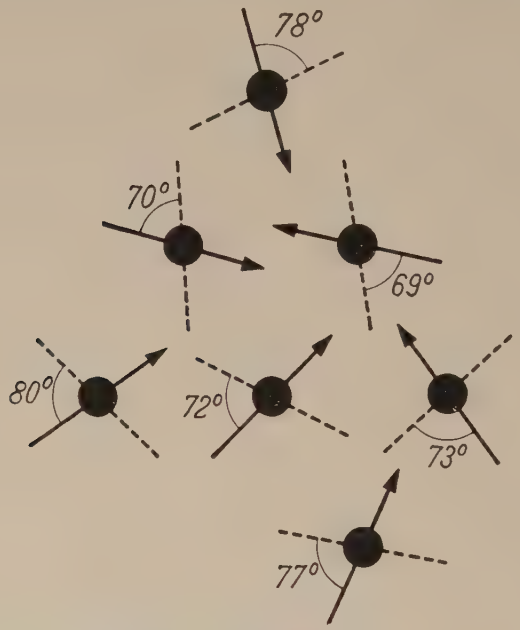

FIGURE 2. The pattern of seven rhabdomeres of the ommatidium in a cross section perpendicular to the axis of the ommatidium. The arrows represent the plane of polarization of the fast beam; the dotted lines, the plane of polarization of the slow beam. Further details are presented in the article by Menzer and Stockhammer.[6]

mounted on the microscope stage so that the eye "looked" at the condenser of the microscope. In this setup, the light coming from the condenser is transmitted by the eye in the "normal" way. It is concentrated by the lens on the sense cells (the rhabdomeres) and passes through them, the only abnormal aspect being that it emerges again where the cells have been cut, instead of being finally absorbed in the pigment at the end of the cell. This emerging light is easily studied in the microscope.

The preparation shows a dark field with small bright points about 2 μ in diameter. These points are the cuts through the rhabdomeres, which transmit and collect the light as wave guides; the sence cells do not show clearly in this type of preparation. The wave-guide effect is discussed in greater detail

below; as to the effects of polarized light, Menzer and Stockhammer[6] found that the rhabdomere, or at least the light-transmitting structure, is double refracting, that is, the refractive index depends on the plane of polarization (for further remarkable details, see the original article). The two authors there suggested a hypothesis to explain the detection of the plane of polarization by the insect eye; however, de Vries[5] holds that their theory meets with serious difficulties, and more recently published papers by Stockhammer[7] do not mention that original theory. The essential objection to it is that the double refraction in the fresh eye is considerably smaller than that which Menzer and Stockhammer found in eyes treated with alcohol and other chemicals. In repeating the experiments on fresh eyes, we have never been able to demonstrate any double refraction. Moreover, although we have looked with all possible care for dichroism,[5] which would appear as a variation in the intensity of transmitted light with rotation of the plane of polarization of the incident light, we were forced to conclude that, if any variation was present, it was certainly less than 1 per cent.

These results are even more interesting in the light of a mechanism suggested by de Vries[5] for the detection of polarization. Since organic molecules tend to absorb visible light if it is polarized in the direction of the (elongated) molecule, the most simple and obvious polarization detector is a sense cell in which all molecules of the photochemical substance are arranged in parallel. By combining the information from two sense cells that are differently oriented, one could reconstruct the plane of polarization of the incident light, because each detector gives the component of the incident beam in its direction. Such a detector is not too far-fetched; it has been shown already that in the vertebrate eye the molecules of the photochemical substance are so arranged that their axis of maximum absorption is perpendicular to the long axis of the cell. This is an optimal arrangement, for the incident light normally propagates along the axis of the cell; consequently, there is no electric vector along the axis, with the result that any molecule having its direction of sensitivity along the axis would never be excited. The light-absorbing portion of the molecules, therefore, is in planes perpendicular to the axis, and it would seem that there is no further orientation *in* these planes; on the contrary, our own experimental evidence[3, 8] indicates that the molecules can move freely in these planes.* By now fixing the molecules in the vertebrate photoreceptor in parallel, our model for the insect eye is realized.

This arrangement should lead to a dichroism of the rhabdomere for light passing through it, but, as already mentioned, dichroism could not be demonstrated. It is not altogether impossible, of course, that the photoreceptors in the median plane of the head, that is, the ocelli, detect the plane of polarization instead of the more obvious lateral eyes of this study. However, a more probable explanation of the discrepancy is a small absorption of light in the rhabdomere, the area in which we have tacitly, and reasonably,

* Recently, W. A. Hagins, in a paper presented at a conference on biophysics, Cambridge, Mass., February, 1958 (to be published), has suggested another explanation for this negative result: if the energy is transmitted first through the structure in the photoreceptor, the bleached molecule need not have the same orientation as the absorbing molecule.

assumed the photochemical substance to be located. If the absorption were about 1 per cent, it might have escaped observation in our measurements of the transmitted light. The insect just "sees" the supposedly small amount of absorbed light, which could very well depend on the plane of polarization. Some evidence for the orientation of material in the rhabdomeres can be derived from the measurements by Menzer and Stockhammer. Although these investigators studied fixed material, this in itself would not have led to an observed and systematic anisotropic arrangement if there had been no anisotropic matrix in the living eye. Furthermore, evidence for a regular arrangement has been obtained recently by electron-microscopic studies, which are summarized by J. J. Wolken elsewhere in this monograph. There is a typical difference between the rhabdomeres of the insect eye and vertebrate photoreceptors. It was only after the conference on which this monograph is based that we heard of the experiments of Baylor and Smith mentioned above, which explain our negative results; obviously the oriented structure of the rhabdomeres has nothing to do with the detection of polarized light.

Optics of the Insect Eye

These observations have led to a correction of some current views on the optics of the ommatidia. Ever since Exner's work on the insect eye,[9] it has been generally assumed that the seven sense cells behind each lens do not "see" different parts of the image. Exner did not describe how he arrived at this conclusion, even though he had originally held the opposite view, that the different cells see different parts of the image just as they do in the vertebrate eye. Nevertheless, his final view, which has come into the literature, is, in our opinion, incorrect.

If the eye is mounted under the microscope as described above, but without a condenser, so that it can look at a point source of light, the resulting phenomenon makes this clear. In the plane where the sense cells are cut, it can be observed that not all the rhabdomeres of one ommatidium transmit light at the same position of the point of light. Instead, they shine one by one when the light is moved, which proves that the visual fields of the different cells vary. Each visual field has a diameter of about 4° and neighboring cells overlap in such a way that the center of one field is approximately on the edge of the field of its neighbor. The field of one ommatidium as a whole has a diameter of about 8°, which means that the visual fields of neighboring ommatidia also overlap appreciably.

The visual field of a single cell is controlled by two factors: the diameter of the rhabdomere (the larger its diameter, the larger the visual field) and the diffraction of light. In the eye itself these two factors have the same order of magnitude. It has been shown that this is the optimal arrangement.[5] Diffraction of light is easily observed in the preparation described above. If the point source is displaced more than 2° from the center of the visual field of a given cell, the rhabdomere shines again, but more weakly, by the diffracted light outside of the first dark ring of the diffraction pattern—the Airy pattern. Electrophysiological studies of the response of the insect eye

also demonstrate a very large field for a single ommatidium—far larger than 8°. It has not yet been determined whether this is due to effects of diffracted light, ineffective screening by the pigment, or nervous interaction.

Sense Cells as Wave Guides

Both the rods and the cones in the vertebrate retina, as well as the rhabdomeres in the insect eye have a refractive index higher than that of the surrounding media. Consequently, light will be totally reflected at the boundaries of these structures and the surrounding media, unless the beam deviates too far from the axis of the cell. As a result of these reflections, radiation is captured by the cell, even when the cell is very long in relation to its diameter. According to O'Brien,[10, 11] Brücke suggested this mechanism more than a century ago.

Depending on the refractive indexes, the internal reflection decreases with an increase in the size of the angle between the incident beam and the axis of the cell. Consequently, the sensitivity of the cell decreases with increasing divergence. This directional sensitivity, which has been found in the human retina, is the so-called Stiles-Crawford effect.[12] These authors found that the threshold of vision increases if the (narrow) incident beam is displaced from the center of the pupil toward its edge. When this occurs the beam falls more obliquely on the retina. A more exact treatment of this phenomenon is given by O'Brien[10, 11] and by Toraldo di Francia.[13]

If the diameter of the wave guide decreases toward its end, as certainly occurs in the case of the retinal cones, the light is concentrated in a smaller region, which implies that a smaller amount of photochemical material is required. It should be emphasized, however, that there are limits to the amount of light concentration possible. The decrease of the size of the cross-section of the wave guide is proportional to the increase in the divergence of the beam. At a certain point the divergence becomes so great that internal reflection no longer occurs.

Integration of Information Obtained by Individual Cells: Further Negative Evidence for the Detection of Polarized Light

The detection of polarized light by individual cells is not in itself polarization vision. The first step in investigating this latter is to construct the plane of polarization from the ratio of the stimuli in adjacent cells having different directions of pigment orientation. The situation is similar to what we know in color vision, whereby each color is characterized by the *ratio* of stimulation of different cones. Still, the most important question remains to be answered; that is, how this information is integrated over the whole visual field. Since we know that the great achievement of color vision is that we see things together with their color at each point of the visual field, we may well ask whether the insect sees its world together with the polarization, or, instead, derives only one item from its information about polarization, namely, the position of the sun. Research in this field could assist study of the anatomy of the optical nerve system to determine whether

special arrangements could be correlated with the computing systems mentioned above.

Some unpublished experiments made in 1952 indicate that it is highly improbable that insects "see" the polarization in each part of the visual field in the same way in which we see colors integrated with the pattern. The experimental arrangement was basically identical with the well-known set up for demonstrating the optomotoric reaction, in which insects are put on a round table surrounded by a cylinder striped vertically in black and white. When this cylinder rotates, the insects tend to follow the moving object. The difference in our setup was that stripes reflecting vertically polarized light were substituted for the white stripes and stripes reflecting horizontally polarized light for the black. No rotation was visible to the naked eye, but through a polaroid the pattern consisted of black and white stripes. The insects showed no optomotoric reaction to this but, when a small difference in intensity between the stripes was introduced, a normal optomotoric reaction was obtained. Of course, this should have made us suspicious about the ability of insects to detect polarized light, but we concluded that the whole situation was so abnormal that the nerve system did not react. We also tried to simulate the polarization pattern of the actual blue sky, but even then the insects did not react.

Finally, another experiment that provided negative evidence for the detection of polarized light is relevant (see also de Vries *et al.*[3] and de Vries[5]). In this the electroretinogram (ERG) of the blowfly was studied in polarized light. The plane of polarization was rotated about 10 times per second (various other frequencies have also been tried). If any polarization effect were present, the ERG could be expected to have a 20 cps component. This was filtered out in a selective way in order to suppress noise from other sources. The sensitivity of the arrangement was demonstrated by the observation that an anisotropy of the incident light of 1 per cent, that is, a difference of intensity of the two directions of polarization of 1 per cent, produced a marked output in the ERG, since this anisotropy is converted into a difference in intensity behind the rotating polaroid. However, when a photomultiplier did not detect any intensity modulation, the ERG did not show any response to rotation of the plane of polarization. Even with refinements, for instance, the illumination of only 1 ommatidium and perhaps even 1 rhabdomere, no response was obtained.

Since this manuscript was prepared before we learned about the work of Baylor and Smith, we have chosen to leave its general form as it was originally. It is obvious, however, that the section on the detection of polarized light now derives its interest mainly from the negative evidence it contains, and from the description of a line of research that started from a wrong assumption.

References

1. von Frisch, K. 1948. Naturwiss. **35**: 12.
2. de Vries, H., R. Jielof & A. Spoor. 1950. Nature. **166**: 958.
3. de Vries, H., R. Jielof & A. Spoor. 1953. Physica. **19**: 419.
4. Autrum, H. 1950. Z. Naturforsch. **5b**: 38.

5. DE VRIES, H. 1956. Progress in Biophysics. **6**: 246. Pergamon Press. London, England.
6. MENZER, G. & K. STOCKHAMMER. 1951. Naturwiss. **38**: 190.
7. STOCKHAMMER, K. 1956. Z. vergleich. Physiol. **38**: 30–83.
8. DE VRIES, H. 1948. Physica. **14**: 345.
9. EXNER, S. 1891. Physiologie der fazettierten Augen. Deutike. Leipzig, Germany & Vienna, Austria.
10. O'BRIEN, B. 1949. J. Opt. Soc. Am. **39**: 12.
11. O'BRIEN, B. 1951. J. Opt. Soc. Am. **41**: 888.
12. STILES, W. S. & B. H. CRAWFORD. 1934. Proc. Roy. Soc. London. **B116**: 55.
13. TORALDO DI FRANCIA, G. 1949. J. Opt. Soc. Am. **39**: 324.

FINE STRUCTURE OF SOME INVERTEBRATE PHOTORECEPTORS

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The first step in the visual process is the absorption of light by a photosensitive visual pigment. Such pigments are often located in specially differentiated parts of the visual receptor cell. The arthropod compound eye, a case in point, is composed of cylindrically shaped ommatidia. The sensory portion of each ommatidium is made up of axially arranged retinular

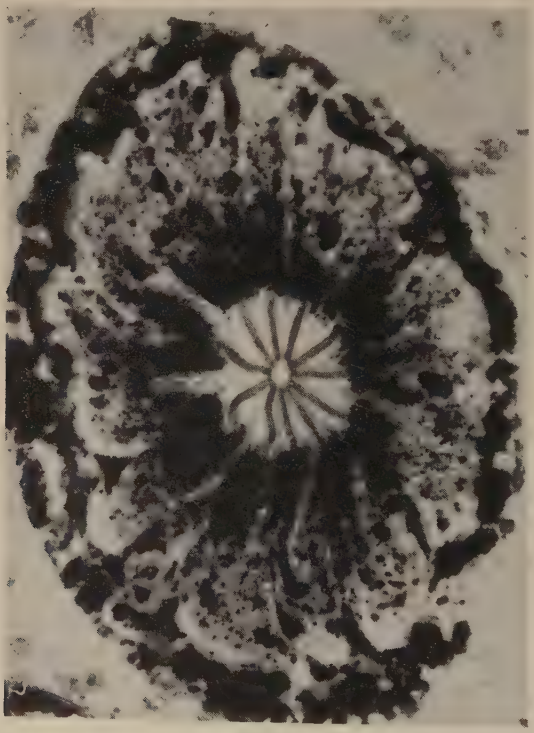

FIGURE 1. Light micrograph of dark-adapted *Limulus* ommatidium in transverse section. The parts of the retinular cells associated with the rhabdom (densely stained spoke-like lines in the center of this section) are relatively clear, being free of pigment.

cells, each contributing a rhabdomere to the rhabdom, which is almost certainly the locus of photosensitive pigment (Wald and Hubbard, 1957). FIGURE 1, which illustrates the ommatidial anatomy, is a light micrograph of a transversely sectioned ommatidium from the compound eye of the horse-

shoe crab, *Limulus polyphemus*. Wedge-shaped retinular cells are symmetrically arranged about the axial canal, which is occupied by the dendritic distal process of a bipolar neuron called the eccentric cell. In this section the rhabdom appears as densely stained spokelike lines radiating from the axial canal. A three-dimensional reconstruction would show it as radiating fins, each composed of the specialized opposing surfaces of two contiguous retinular cells. The zones of division between retinular cells are in axial longitudinal planes bisecting the rhabdomal fins; thus the contribution of each retinular cell to the rhabdom is a V-shaped rhabdomere. In the dark-adapted *Limulus* eye the central core of the ommatidium is clear, allowing

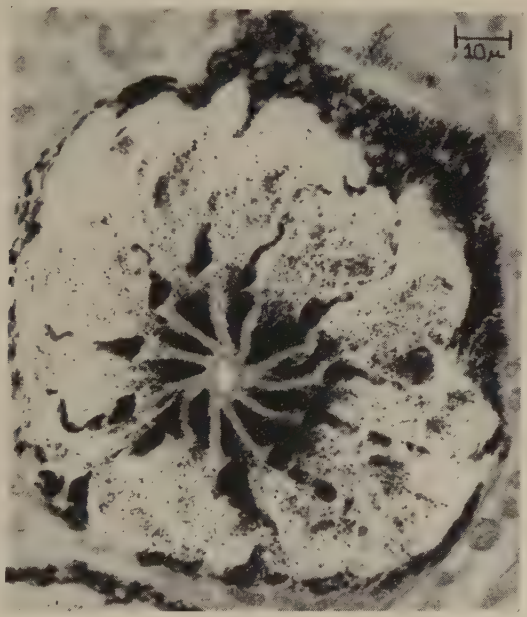

FIGURE 2. Light micrograph of light-adapted *Limulus* ommatidium in transverse section. In this fully light-adapted ommatidium, pigment granules have migrated into the areas between the rhabdom's fins.

entering light waves easy access to the rhabdom (FIGURE 1). However, in the fully light-adapted eye (FIGURE 2) pigment granules have migrated from the periphery of the retinular cells into the areas between the fins of the rhabdom.

Investigation of the fine structure of arthropod rhabdomeres has shown that they resemble a honeycomb on a very small scale (Miller, 1957a; Goldsmith and Philpott, 1957; Wolken *et al.*, 1957; Miller, 1957b). The tubular units of this honeycomblike structure are tightly packed microvilli of the retinular cell's surface. In *Limulus*, the long axes of these microvilli lie in transverse planes; consequently transverse sections have a banded appearance as shown in the electron micrograph, FIGURE *3a*. The horizontal mark-

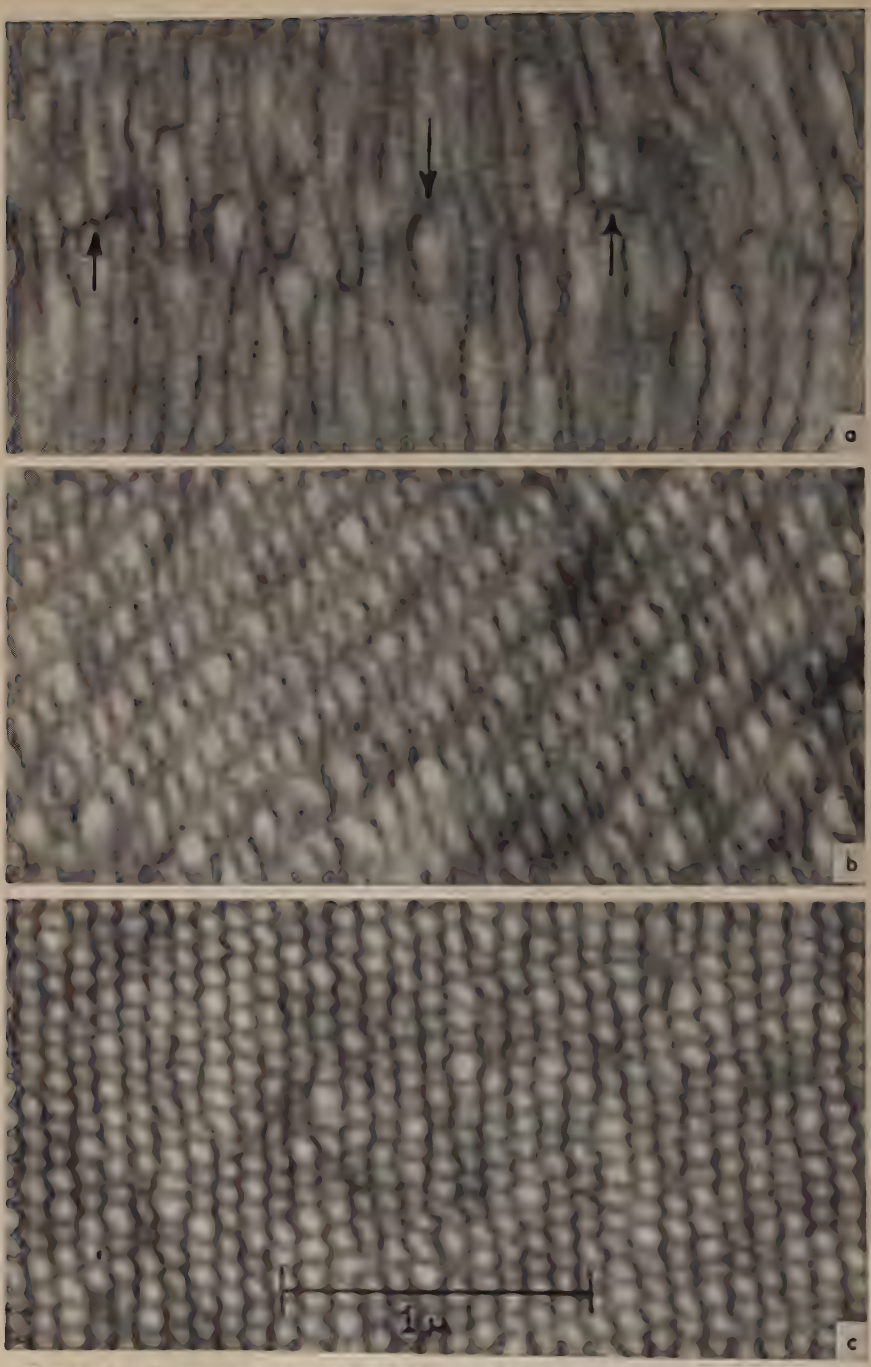

FIGURE 3. Electron micrographs of rhabdom of *Limulus* compound eye: (a) transverse section showing the tightly packed microvilli of the retinular cell's surface, and the horizontal markings (arrows) at the junction of the closed ends of the microvilli from neighboring retinular cells; (b) oblique section; and (c) a section perpendicular to the long axes of the tubelike microvilli.

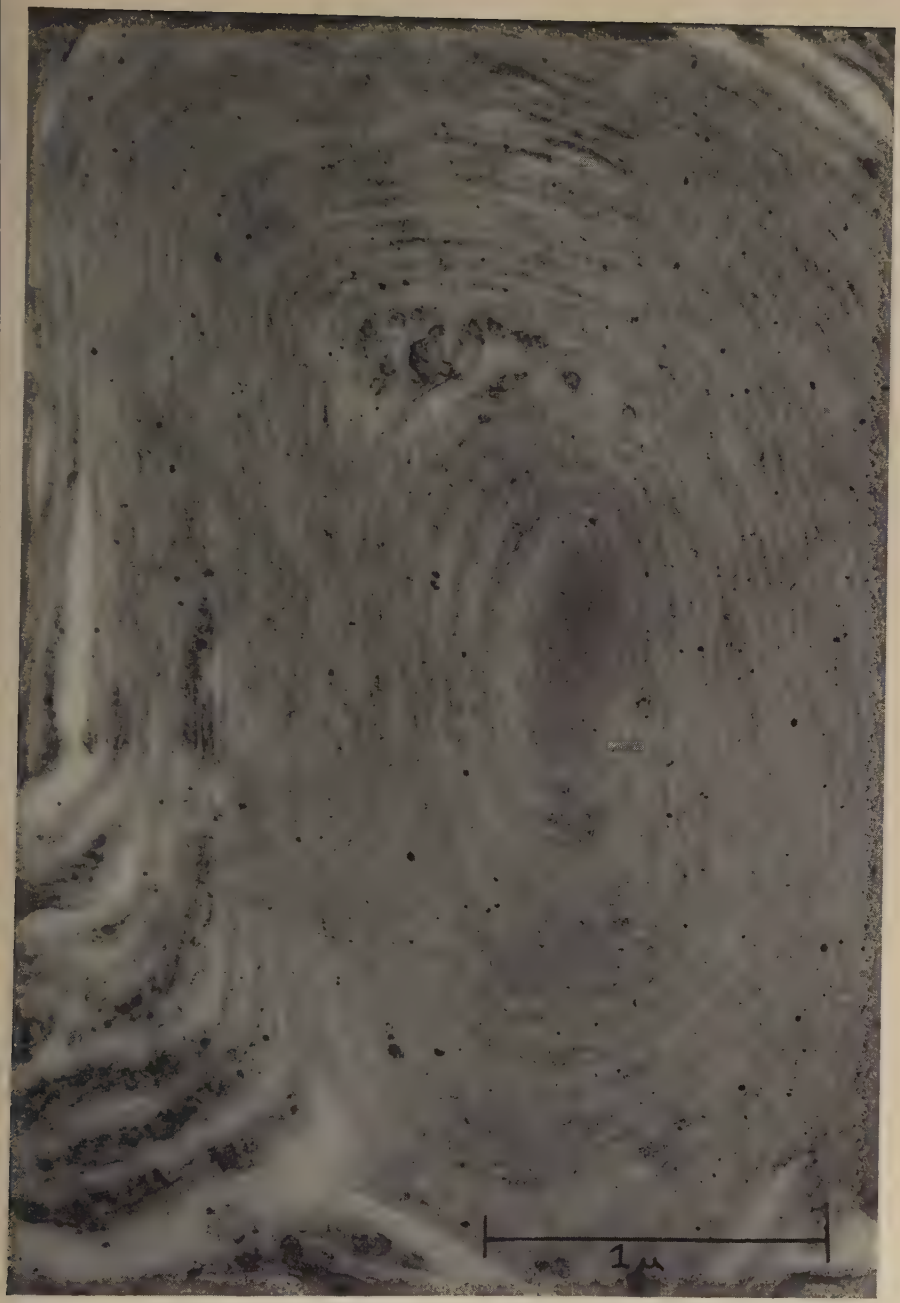

FIGURE 4. Electron micrograph of the distal sense cell appendage in the eye of the bivalved mollusk, *Pecten*. The appendage appears as concentrically arranged alternately lightly and densely stained bands, which are assumed to be lamellae. These lamellae are continuous with the stalks of cilia, parts of which are seen in the lower left corner of the micrograph.

ings in this figure indicated by arrows, are in the zone of abutment of the microvilli of neighboring retinular cells. FIGURE 3*b* is an electron micrograph of a *Limulus* rhabdomere in oblique section in which the oblong outlines of the microvilli are seen, and FIGURE 3*c* is an electron micrograph showing the appearance of microvilli that have been cut squarely across

In vertebrate eyes, also, the light receptor cells—the rods and cones—have specially differentiated parts, the outer segments, which contain visual pigments. The rod outer segment, as Sjöstrand (1953) has demonstrated by means of the electron microscope, is a stack of double-membrane disks, and it has recently been shown by De Robertis (1956) and by Porter (1957) that the outer segment is derived from a cilium, an elementary cellular structure that is utilized similarly in the development of several other sense organs as well. With respect to the arthropod rhabdomere, no evidence of a ciliary derivation has been observed. However, cilia in arthropods, if not entirely lacking, are at least very rare. Most of the other animal phyla, nevertheless, are richly endowed with cilia. We are currently investigating the eye of the bivalved mollusk, *Pecten*, and have found there specialized structures that are clearly derived from cilia and are thought to be photoreceptors.

Pecten, the common scallop, has about one hundred eyes, each a millimeter in diameter; half of these are located along the mantle fringe of each valve. Each eye has a double retina. There is a proximal layer of rodlike cells, the axons of which comprise the proximal ramus of the optic nerve, and also a layer of distal sense cells having axons that form the distal ramus. By investigating the responses of the branches of the optic nerve, Hartline (1938) found that the proximal retina responds to steady illumination of the eye with a steady discharge of nerve impulses, while the distal retina discharges impulses only in response to a decrease in illumination. Electron microscopic investigation of the cell thought to be responsible for this "off" response, the distal sense cell, shows that it has globular appendages composed of concentrically arranged lightly and densely stained bands (FIGURE 4), which are interpreted as representing lamellae. It is thought that these appendages are photoreceptor structures. Electron micrographs show, furthermore, that the lamellae of the appendages are continuous with structures that have been clearly identified as the stalks and basal bodies of cilia (Miller, 1958).

Comparative electron microscopic investigations of photoreceptors are thus revealing a diversity of ultrastructure: stacks of membranes in the vertebrate, microvilli in the arthropod, and concentric lamellae in *Pecten*, a mollusk. Two of these photoreceptor types, the vertebrate rod outer limb and the *Pecten* distal sense cell appendage, are embryologically related in that they are both derived from cilia. All of these specially differentiated sense cell organelles have a common feature: they present a series of membranes to an incoming light wave.

References

DE ROBERTIS, E. 1956. J. Biophys. Biochem. Cytol. **2**(4) Suppl.: 209.
GOLDSMITH, T. H. & D. E. PHILPOTT. 1957. J. Biophys. Biochem. Cytol. **3**(3): 429.

Hartline, H. K. 1938. J. Cellular Comp. Physiol. **3**: 465.
Miller, W. H. 1957a. J. Biophys. Biochem. Cytol. **3**(3): 421.
Miller, W. H. 1957b. Science. (Abstr.) **126**(3285): 1233.
Miller, W. H. 1958. J. Biophys. Biochem. Cytol. **4**(2): 227.
Porter, K. R. 1957. Harvey Lectures. Ser. **51**: 175.
Sjöstrand, F. S. 1953. J. Cellular Comp. Physiol. **42**: 15.
Wald, G. & R. Hubbard. 1957. Nature. **180**: 278.
Wolken, J. J., J. Capenos & A. Turano. 1957. J. Biophys. Biochem. Cytol. **3**(3): 441.

NEURAL INTERACTION IN THE EYE AND THE INTEGRATION OF RECEPTOR ACTIVITY

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The neural mechanisms in the retina are more than mere devices for the passive transmission of information about the temporal and spatial patterns of illumination on the receptor mosaic. Certain significant features are selected from the immense detail there, enhanced at the expense of other less significant features, and only then transmitted to more central parts of the nervous system. Just as the artist often seeks to avoid the "danger in becoming engrossed in accuracy at the expense of significance" (Warner, 1952), so does the eye sacrifice accuracy about information of little consequence in order to enhance features that are most significant to the organism.

Among the significant features of a visual scene are the loci of transitions from one color to another or from one intensity to another. As long as contours are indicated, by whatever means—even by the thin line of a line drawing or cartoon—much of the essential information in a visual scene is retained.

How is information about such contours abstracted from information in the retinal image and transmitted by the optic nerve? In addition to the simple and direct signaling of intensity levels on the various receptors in the receptor mosaic, there are, apparently, highly specialized integrative mechanisms that accentuate abrupt gradients. The myriad interconnections in the retina form a nervous center with a complexity as great as that of a comparable portion of the brain, and the interplay of excitatory and inhibitory influences over these interconnections yields patterns of optic nerve activity that are not direct copies of the pattern of external stimulation.

Two important consequences of this integrative action are: (1) the enhancement of differences in neural activity from differently illuminated regions of the retina, and (2) the enhancement of neural responses to temporal changes in intensity. This paper examines these processes both in an invertebrate eye and in the more complex retinas of vertebrates.

In the compound eye of *Limulus* the structural organization is far simpler than it is in the vertebrate eye. Nevertheless, Hartline (1949) found that the sensory elements, that is, the ommatidia, in this eye do exert marked influences upon one another: this interaction is purely inhibitory and is undoubtedly mediated by a plexus of nerve fibers that interconnect the bundles of axons originating in the ommatidia (Hartline *et al.*, 1956).

Each ommatidium (Miller, 1957) contains roughly a dozen cells: a cluster of wedge-shaped retinular cells and one bipolar neuron, the eccentric cell. Both the eccentric cell and the retinular cells have axons, which form a bundle that joins with nerve bundles from other ommatidia to become the optic nerve. The axons of both the eccentric and the retinular cells branch profusely, and these branches, which are for the most part less than a micron in

diameter, constitute the plexus of nerve fibers that interconnects the nerve bundles emanating from the proximal portions of the ommatidia (FIGURE 1). FIGURE 2 shows a retinular cell axon and branch *B*; FIGURE 3b shows a fine axonal branch *B* from an eccentric cell axon *E*. These fine branches converge in regions of neuropile (*N*, in FIGURES 3a and b) in close proximity to eccentric cell axons *E*. In the regions of neuropile, the roughly circular outlines of the axonal branches can be seen; within these fine branches are

FIGURE 1. Light micrograph of a section of the compound eye of *Limulus* taken perpendicular to the cornea and stained with silver by the method of Samuel (1953). The cornea has been removed. The heavily pigmented sensory parts of the ommatidia are at the top of the figure. The silver-stained nerve fibers originating in the retinular cells and the eccentric cell of each ommatidium emerge as a bundle from the proximal end of each ommatidium and join with similar bundles from other ommatidia to form the optic nerve, part of which is seen at the bottom of the figure. The axons in these bundles branch profusely and these small axonal branches form the lateral network of the plexus. Reproduced by permission from Hartline *et al.* (1956).

circular outlines about 300 Å in diameter (FIGURE 3c). These vesicular structures resemble the synaptic vesicles described by Palade (1954), by De Robertis and Bennett (1955), and by others. For this reason the neuropile described here is thought to be a synaptic region. The fine axonal branches and the regions of neuropile in which they converge provide an anatomical basis for the inhibitory interaction in this eye.

The method of studying inhibitory interaction in the eye of *Limulus* has been described elsewhere (Hartline *et al.*, 1956). Action potential spikes are recorded from a single fiber dissected from the optic nerve, as illustrated

schematically in FIGURE 4. Responses to light are elicited in such a fiber only by illuminating the particular ommatidium from which it arises; the frequency of the discharge of impulses depends primarily upon the intensity of illumination on the ommatidium. However, the frequency of discharge from a particular ommatidium is significantly reduced by stimulation of neighboring elements. The magnitude of this inhibition increases with an increasing intensity of illumination on the neighboring ommatidia. It is also greater, the larger the number of neighboring ommatidia that are illuminated, and the closer they are to the particular ommatidium under observation.

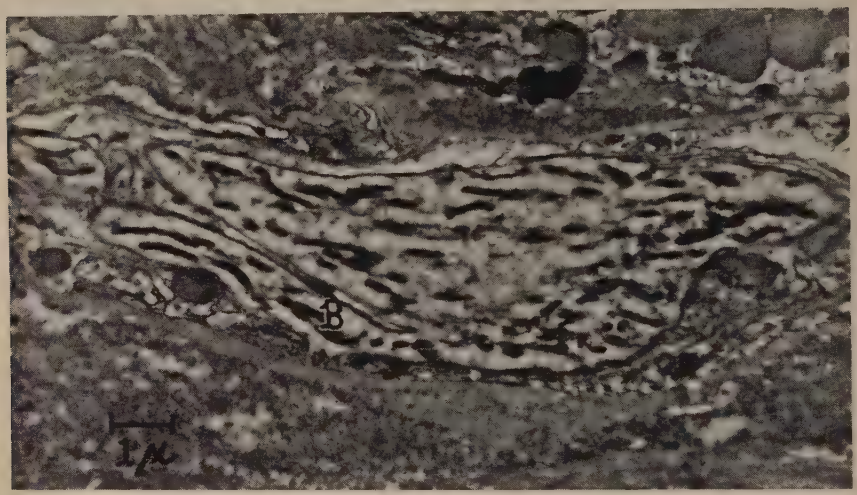

FIGURE 2. Electron micrograph of retinular cell axon and axonal branch. The outline of this obliquely sectioned retinular cell axon is roughly oval. A small axonal branch is labeled *B*. The dark-stained rod-shaped objects within the axon are mitochondria. The tissue from which this micrograph and those in FIGURE 3 were made was fixed at 4° C. for one hour in a solution 1 per cent OsO_4, 0.5 per cent $K_2Cr_2O_7$, and 0.75 M sucrose at *p*H 7.7.

These inhibitory influences are exerted mutually among the receptors in the eye of *Limulus*: each ommatidium is a neighbor of its neighbors. If activity is recorded from two optic nerve fibers coming from two ommatidia not too widely separated in the eye, the frequency of the maintained discharges of impulses in response to steady illumination is lower when both ommatidia are illuminated together than when each is illuminated by itself (Hartline and Ratliff, 1957). The magnitude of the inhibition of each one has been shown to depend only on the degree of activity of the other; thus the activity of each is the resultant of the excitation from its respective light stimulus and the inhibition exerted on it by the other. Furthermore, it has been shown that the inhibition exerted on each is a linear function of the degree of activity of the other (FIGURE 5).

The responses to steady illumination of two receptor units that inhibit each other mutually are described quantitatively by two simultaneous linear

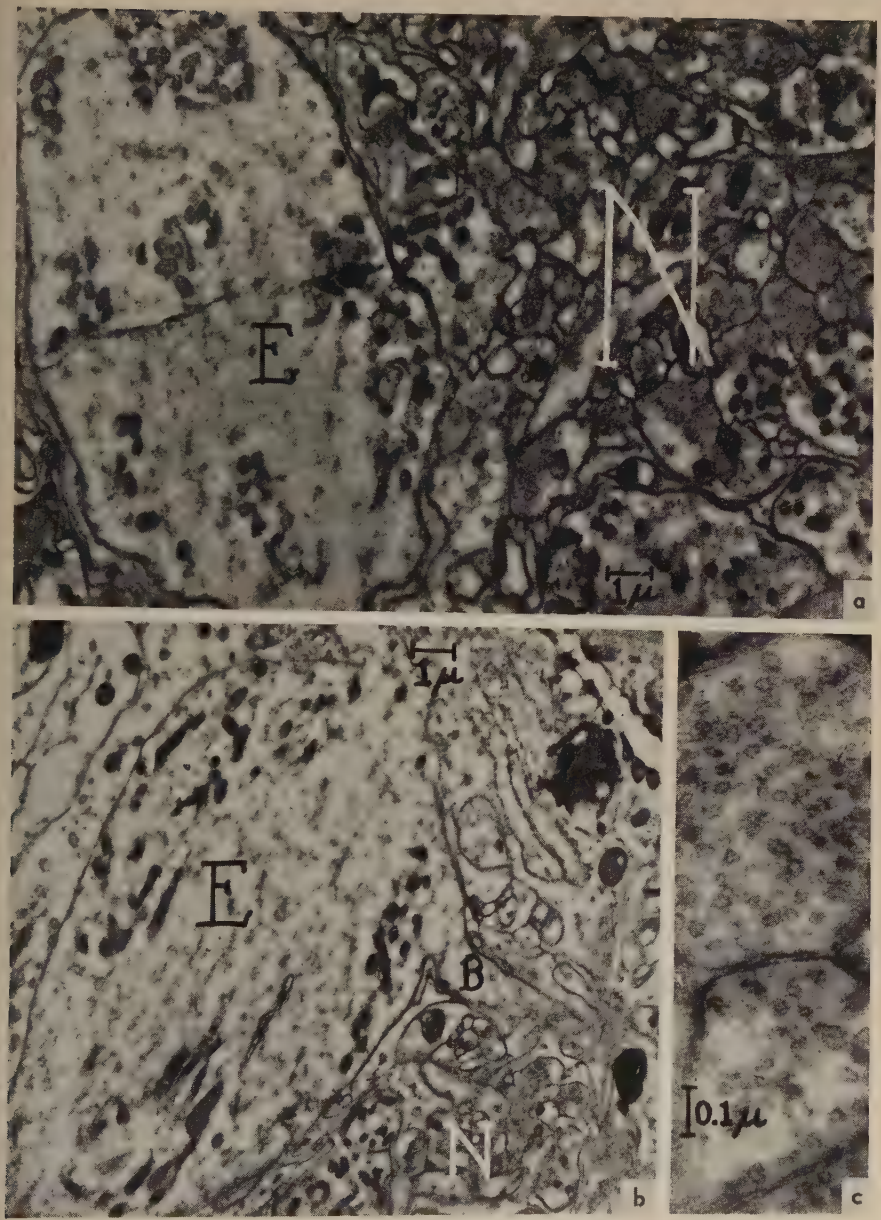

FIGURE 3. Electron micrographs showing (a) an eccentric cell axon *E* and a region of neuropile *N*. The neuropile is formed by the convergence of axonal branches of retinular and eccentric cell fibers. These branches are seen as circular outlines about 1 μ in diameter. (b) Eccentric cell axon *E*, with axonal branch *B* and region of neuropile *N*. (c) Part of neuropile at high magnification. Most of two axonal branches can be seen. Within these branches are many dark-bordered circular outlines about 300 Å in diameter, the synaptic vesicles.

equations that express concisely all the features of the interaction. The activity of the receptor unit—its response r—is to be measured by the frequency of discharge of impulses in its axon. This response is determined by the excitation e supplied by the external stimulus to the receptor, diminished by whatever inhibitory influences may be acting upon the receptor as a result of the activity of neighboring receptors. It should be noted that the excitation of a given receptor is to be measured by its response when it is illuminated by itself, thus lumping together the physical parameters of the

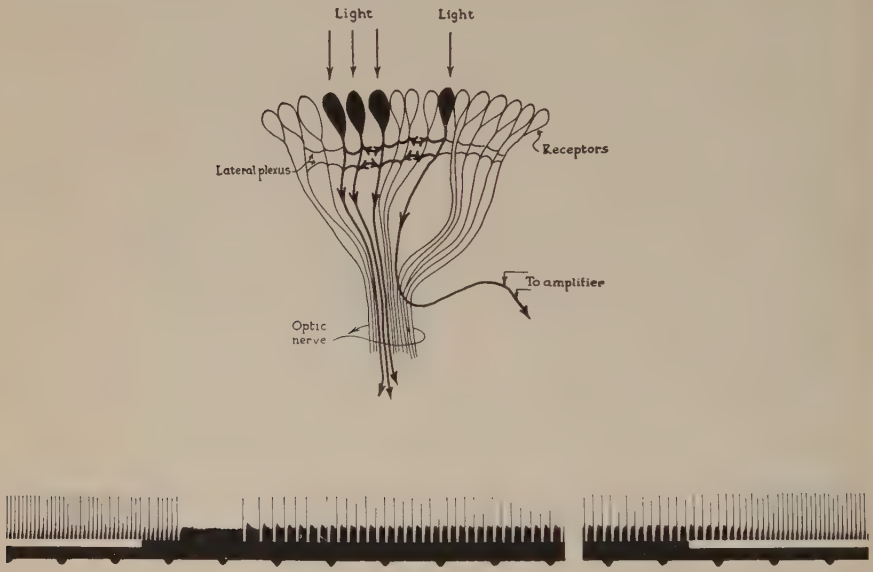

FIGURE 4. Inhibition of the activity of a steadily illuminated ommatidium in the eye of *Limulus*, produced by illumination of a number of other ommatidia near it. The experimental arrangement is indicated in the diagram above; below is an oscillographic record of the discharge of impulses in the optic nerve fiber from the steadily illuminated ommatidium. The blackening of the white line above the time record signals the illumination of the neighboring ommatidia. Time in one-fifth sec. Record reproduced by permission from Hartline *et al.* (1956).

stimulus and the characteristics of the photoexcitatory mechanism of the receptor. Each of two interacting receptor units inhibits the other to a degree that depends linearly on its own activity. The responses of two such units are therefore given by a pair of simultaneous equations:

$$r_1 = e_1 - K_{1,2}(r_2 - r^0{}_{1,2})$$
$$r_2 = e_2 - K_{2,1}(r_1 - r^0{}_{2,1})$$

The subscripts are used to label the respective receptor units. In each of these equations the magnitude of the inhibitory influence is given by the last term, which is written in accordance with the experimental findings as a simple linear expression. The "threshold" frequency that must be exceeded

before a receptor can exert any inhibition is represented by r^0. The "inhibitory coefficient" K is labeled in each equation to identify the direction of the action: $K_{1,2}$ is a coefficient of the action of receptor 2 on receptor 1; $K_{2,1}$ vice versa.

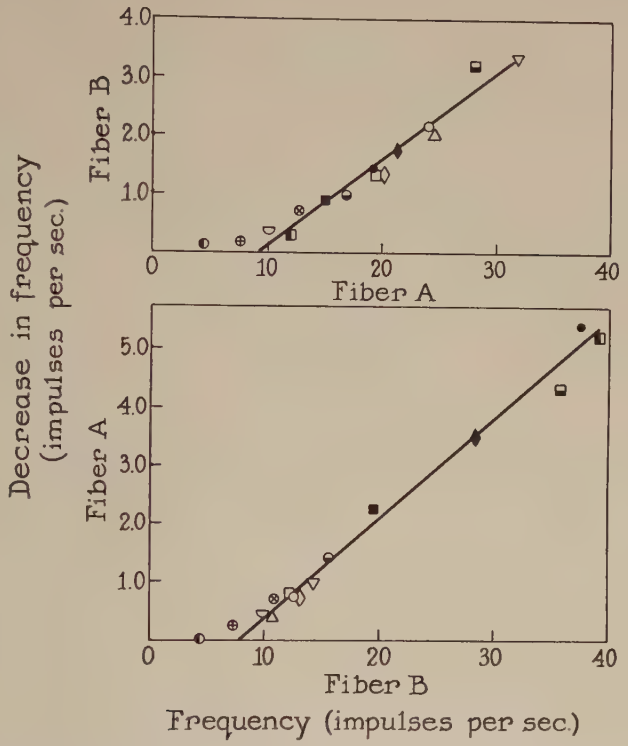

FIGURE 5. Graphs showing mutual inhibition of two receptor units, A and B, 1 mm. apart in the lateral eye of *Limulus*. Action potentials were recorded from two optic nerve fibers simultaneously when the respective ommatidia from which these fibers originated were illuminated. In each graph the magnitude of the inhibitory action (decrease in frequency of impulse discharge) of one of the ommatidia is plotted on the ordinate as a function of the degree of concurrent activity (frequency) of the other on the abscissa. The different points were obtained by using various intensities of illumination on ommatidia A and B, in various combinations. The data for points designated by the same symbol were obtained simultaneously. In the upper graph the slope of the line gives the value of the inhibitory coefficient of the action of receptor A on receptor B, $K_{BA} = 0.15$; the intercept of the line with the axis of abscissas gives the value of the threshold $r^0_{BA} = 9.3$ impulses per second. From the lower graph, $K_{AB} = 0.17$; $r^0_{AB} = 7.8$ impulses per second. Reproduced by permission from Hartline and Ratliff (1957).

The magnitudes of the inhibitory coefficients and the values of the thresholds depend on the separation of the units in question (FIGURE 6). The smaller this separation, the greater are the inhibitory coefficients and the lower the thresholds of action (Ratliff and Hartline, 1957). Experiments to be described elsewhere show that these differences are not due to different

properties of the elements themselves, but do in fact depend upon distance. These results mean that no special term is required in the above equations for distance per se; all the effects attributable to distance are included in the terms for the thresholds and the inhibitory coefficients.

The quantitative description given thus far is concerned only with the interaction of two elements. To extend the description to more than two elements it is necessary to know how the inhibitory influences from different elements combine with one another. This information may be obtained by illuminating groups of receptors on either side of a "test" receptor whose

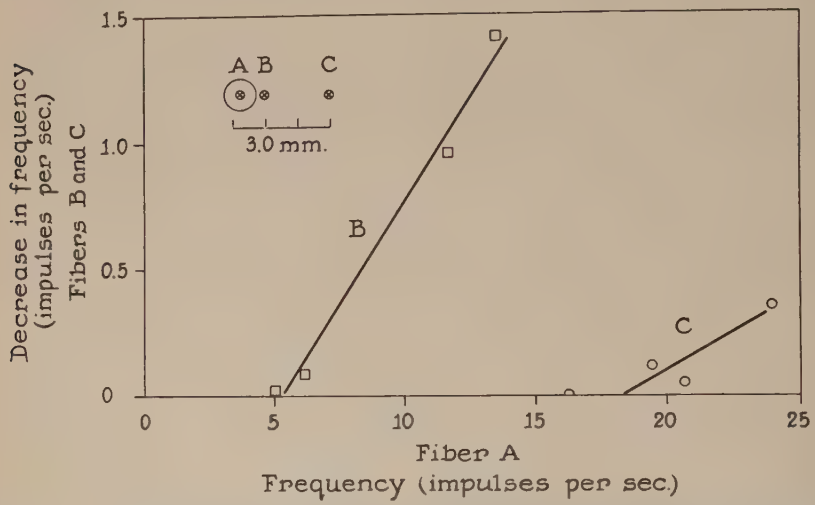

FIGURE 6. The decrease of inhibition with increasing distance between receptors in the lateral eye of *Limulus*. The magnitude of the inhibition, the decrease in frequency, produced by a small group of receptors A on a nearby receptor B and on a more distant receptor C is plotted as ordinate; the frequency of response of one of the ommatidia in the center of the small group A is plotted as abscissa. The spatial arrangement of the ommatidia relative to one another is indicated on the figure. The coefficient of inhibitory action of A on the nearby ommatidium B (K_{BA}) was far greater than was the coefficient of action of A on the distant ommatidium C (K_{CA}), and the threshold of inhibitory action of A on B (r^0_{BA}) was considerably smaller than was the threshold of action of A on C (r^0_{CA}).

activity is measured. If the groups are placed sufficiently far apart to exert no inhibitory influences upon each other, yet close enough to the test receptor to exert measurable effects upon it, the inhibitory effects they produce will be undistorted by their own mutual inhibition. Under these conditions the experimental results are quite simple. The inhibitory effects, when measured in terms of the decrease in frequency of the test receptor, combine in a simple additive manner: the arithmetical sum of the inhibitory effects that each group produces separately equals the physiological sum obtained by illuminating the two groups together (Hartline and Ratliff, 1958).

These results permit the extension of the quantitative description to include any number of interacting elements by expressing the total inhibition

exerted on any one receptor as the arithmetical sum of the individual inhibitory contributions from all the others. The set of simultaneous equations for n interacting receptors may be constructed by writing n equations, each with $n - 1$ inhibitory terms combined by simple addition:

$$r_p = e_p - \sum_{j=1}^{n} K_{p,j}(r_j - r^0{}_{p,j})$$

where $p = 1, 2 \ldots n; j \neq p$; and $r_j \not< r^0{}_{p,j}$.*

These equations have been applied to experimental results obtained by illumination of three receptors, from one of which activity was recorded, or of three groups of receptors, all close to one another and all interacting to various degrees. Depending on the sizes of the groups and their distances from one another, different degrees of interaction were obtained in various combinations. A corresponding variety of quantitative relations among the inhibitory effects has been observed; each of these is adequately accounted for by expressions derived from the above set of equations (Hartline and Ratliff, 1958).

There is considerable direct electrophysiological evidence that a lateral inhibitory mechanism similar to the one just described in the eye of *Limulus* exists in the more complex vertebrate retina. Hartline (1939), Barlow (1953), and Kuffler (1953), recording the discharge of impulses from single retinal ganglion cells, have all described effects that reveal inhibitory interaction within the vertebrate retina. Such effects, however, are considerably complicated by an interplay of inhibitory and excitatory influences, which result in great diversity and often lability of the patterns of optic nerve activity.

Inhibitory interaction can achieve important visual effects. In an animal's normal environment different receptors of the eye are usually subjected to unequal intensities of illumination from different parts of the visual field. The more intensely illuminated retinal regions exert a stronger inhibition on the less intensely illuminated ones than the latter do upon the former. As a result, differences in neural activity from differently lighted retinal regions are exaggerated; in this way contrast is enhanced.

Such an interacting inhibitory mechanism is probably the basis of a number of well-known phenomena of brightness contrast and color contrast. In human vision, many of the familiar properties of simultaneous brightness contrast can be explained by postulating a similar inhibitory interaction in the visual pathways (Fry, 1948). If the inhibitory influence is assumed to decrease with increasing distance between retinal regions, border contrast and related effects such as Mach's bands may also be explained. All these effects can be interpreted as the result of the action of inhibitory mechanisms that exaggerate differences in the neural responses to the various colors and intensities of illumination over the retinal mosaic—especially in these regions of the retinal image where the variations are abrupt. In this manner the

* Hartline and Ratliff (1958) describe restrictions on the range of these equations.

information about contours is preserved and enhanced at the expense of accuracy of information about intensity and wave length.

It is a fundamental characteristic of many receptors that they not only signal information about steady stimulus conditions, but that they also respond with accentuated changes in activity to sudden changes in stimulus intensity. For example, in the single receptor unit of the *Limulus* eye a large transient increase in the frequency of discharge of nerve impulses is produced

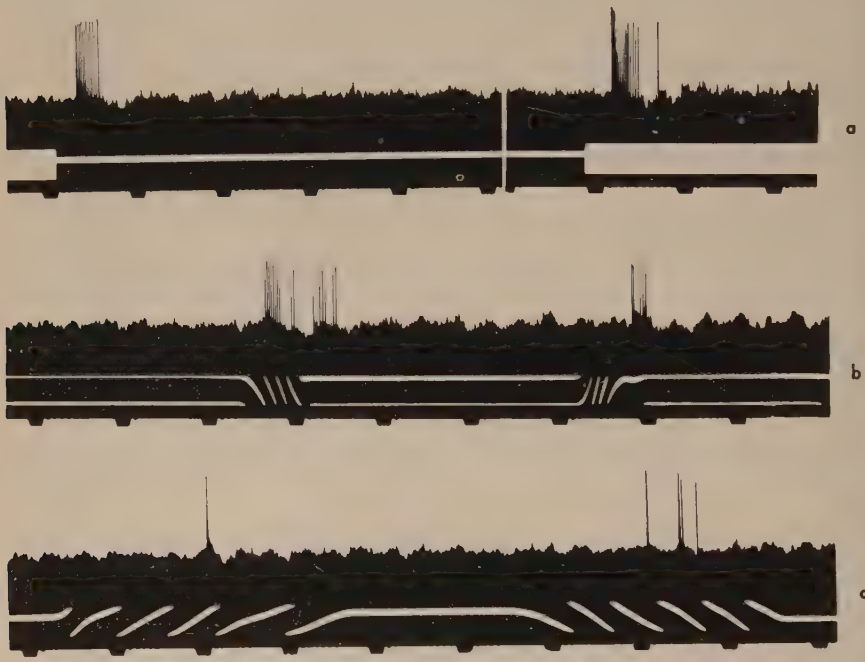

FIGURE 7. Oscillograms of action potentials in a single optic nerve fiber of the frog, showing responses to slight movements of a small spot of light 50 μ in diameter on the retina. Fiber responded at "on" and "off"; there was no discharge during steady illumination if the stimulus spot was stationary as in a. The signal marking a period of illumination blackens the white strip above the one-fifth-sec. time marks. Slight movements of the stimulus spot elicited short bursts of impulses as in b and c. Movements of the spot on the retina are signaled by narrow white lines appearing above the time marker; each line corresponds to 7 μ on the retina. Reproduced by permission from Hartline (1940).

in response to a small increase in level of illumination, the frequency eventually subsiding to a steady level only slightly greater than that preceding the change in illumination. A small decrease in intensity is likewise followed by a large decrease in frequency, after which there is gradual recovery to a level slightly below that existing prior to the decrease in illumination (MacNichol and Hartline, 1948).

The vertebrate eye signals transients in a more highly specialized manner. Indeed, many retinal elements discharge impulses only in response to changes

in illumination. Some elements respond both to onset and cessation of illumination, others only to one or the other (Hartline, 1938; Granit, 1955; and Kuffler, 1953). In the particular axon whose responses are shown in FIGURE 7, impulses appeared only when the light went on and again when it went off. Responses of such an element may also be elicited by moderate increases and decreases in the illumination, or even by moving a heterogeneous field of illumination across the receptor mosaic. The vigor of the response depends on the magnitude and abruptness of the change. Such "on-off" mechanisms must be of considerable significance in the detection of

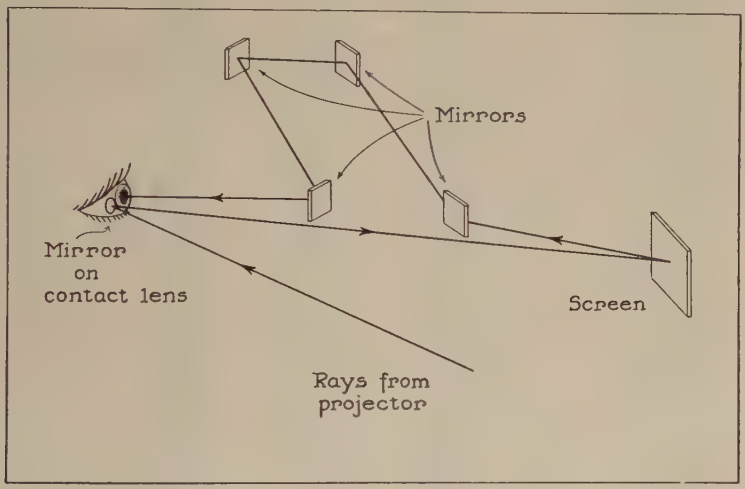

FIGURE 8. Diagram of a method for counteracting the effects of eye movements. A contact lens bearing a small mirror, which is worn by the subject, follows the movements of the eye. An image of a test pattern is projected on a screen by way of this mirror on the contact lens. After a few seconds the details of the pattern fade from view. The viewing path through the system of mirrors is double the distance from the eye to the screen, thus compensating for the fact that the angle of rotation of the beam reflected from the contact lens mirror is double the angle of rotation of the eye itself. Under these conditions the retinal image is stationary with respect to the receptor mosaic. Redrawn after Riggs *et al.* (1953). Compare Ditchburn and Ginsborg (1952).

contours. As an object moves in the field of view, or as the eye moves in scanning a heterogeneous field, vigorous neural responses are elicited from elements in those parts of the retina on which the illumination changes.

That such motions actually play an important role in human vision has been shown in experiments in which an optical device (FIGURE 8) is used to cancel the effects of all eye movements. When a retinal image is formed that is stationary with respect to the receptor mosaic, all contours and discontinuities gradually fade out, and within a few seconds the visual field appears uniform, although the image is physically unchanged on the retina (Riggs *et al.*, 1953). If the image is flickered (Cornsweet, 1956) or is caused to move on the retina (Krauskopf, 1957), it immediately reappears. These phenomena are not an artifact of the necessary attachments to the eye, as

FIGURE 9. Oscillograms of diverse "types" of impulse discharge patterns in single fibers of *Limulus* optic nerve. The typical response to steady illumination of a single ommatidium is a sustained discharge. In the records shown the receptor was illuminated simultaneously with other nearby receptors, which exerted inhibition upon it. Depending on the conditions of illumination, various kinds of responses can be "synthesized." (a) Records of synthetic on-off responses produced by two different durations of illumination; approximately 1 sec. was cut from the middle of each record. (b) Records of synthetic off responses for various durations of illumination. In the lower record of (b) the off response, comparable to that in the upper record, was inhibited by reillumination. Time is marked in fifths of a second. Signal of exposure to light blackens the white line above the time marks. Note that the responses are less vigorous than those responses, which they imitate, of vertebrate optic nerve fibers such as are shown in FIGURE 7. Compare Ratliff and Miller (1957).

may be shown by the use of methods for producing stabilized retinal images that do not require such attachments, for example, as described by Ratliff (1958).

It would seem, then, that two more or less distinct mechanisms play important roles in the detection and signaling of information about contours: (1) a "static" inhibitory mechanism that serves to exaggerate information about *spatial* differences in intensity, and (2) a "dynamic" mechanism that serves to signal information about *temporal* changes in intensity. Recent observations, however, suggest that the second of these mechanisms may be related closely to the first.

The relatively simple sustained discharge typical of the optic nerve fibers of *Limulus* may be greatly modified by various means. First, a pronounced afterdischarge may be obtained by proper control of exposure time, intensity of the stimulus, and state of adaptation. Second, as described above, the frequency of the discharge of impulses from a particular ommatidium may be decreased by illuminating neighboring ommatidia. By pitting these excitatory and inhibitory influences against one another Ratliff and Mueller (1957) have synthesized on-off and off responses in individual fibers of the *Limulus* optic nerve.

These responses (FIGURE 9) possess most of the properties of the analogous responses in the vertebrate eye: the on-off responses are characterized by a burst of activity when the light is turned on, no further activity as the light stays on, and a final burst of activity soon after the light is turned off. The off responses also have the properties of the vertebrate off response: no discharge appears until after the light goes off, and the discharge may be inhibited by reillumination. These experiments lend support to the view expressed by Granit (1933) and by Hartline (1938) that on-off and off responses of vertebrate retinas are the result of the complex interplay of excitatory and inhibitory influences.

It is evident that the optic nerve does not consist of a collection of "private lines" with which to transmit with perfect fidelity information about the distribution of radiant energy falling on the receptor mosaic. The pattern of activity in the optic nerve is by no means a direct copy of the pattern of radiant energy incident on the retina. Due to the interplay of excitatory and inhibitory influences in the retina, certain information of special significance to the organism is accentuated at the expense of less significant information.

References

BARLOW, H. B. 1953. J. Physiol. **119**: 69.

CORNSWEET, T. N. 1956. J. Opt. Soc. Am. **46**: 987.

DE ROBERTIS, E. D. P. & H. S. BENNETT. 1955. J. Biophys. Biochem. Cytol. **1**(1): 47.

DITCHBURN, R. W. & B. L. GINSBORG. 1952. Nature. **170**: 36.

FRY, G. A. 1948. Am. J. Optom. and Arch. Am. Acad. Optom. **25**: 162.

GRANIT, R. 1933. J. Physiol. London. **77**: 207.

GRANIT, R. 1955. Receptors and Sensory Perception. Yale Univ. Press. New Haven, Conn.

HARTLINE, H. K. 1938. Am. J. Physiol. **121**: 400.

HARTLINE, H. K. 1939. Am. J. Physiol. **126**: 527.

HARTLINE, H. K. 1940. Am. J. Physiol. **130**: 690.

HARTLINE, H. K. 1949. Federation Proc. **8**: 69.
HARTLINE, H. K. & F. RATLIFF. 1958. J. Gen. Physiol. **41**: 1049.
HARTLINE, H. K., H. G. WAGNER & F. RATLIFF. 1956. J. Gen. Physiol. **39**: 651.
HARTLINE, H. K. & F. RATLIFF. 1957. J. Gen. Physiol. **40**: 357.
KRAUSKOPF, J. 1957. J. Opt. Soc. Am. **47**: 740.
KUFFLER, S. W. 1953. J. Neurophysiol. **16**: 37.
MACNICHOL, E. F. & H. K. HARTLINE. 1948. Federation Proc. **7**: 76.
MILLER, W. H. 1957. J. Biophys. Biochem. Cytol. **3**(3): 421.
PALADE, G. E. 1954. Anat. Record. (Abstr.) **118**: 335.
RATLIFF, F. & H. K. HARTLINE. 1957. Science. **126**: 1234.
RATLIFF, F. & C. G. MUELLER. 1957. Science. **126**: 840.
RATLIFF, F. 1958. J. Opt. Soc. Am. **48**(4): 274–275.
RIGGS, L. A., F. RATLIFF, J. C. CORNSWEET & T. CORNSWEET. 1953. J. Opt. Soc. Am. **43**: 495.
SAMUEL, E. P. 1953. Anat. Record. **116**: 511.
WARNER, L. 1952. The Enduring Art of Japan. Harvard Univ. Press. Cambridge, Mass.

Part II. Biochemistry of Photoreception

ON THE VISUAL SYSTEM OF THE BEE (*APIS MELLIFERA*)*

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During the past few years there has been considerable interest in the comparative biochemistry of vision. Visual pigments from a variety of vertebrates,[1, 2] the squid,[3] and the lobster[4] have been described. These comparative studies reveal a common biochemical basis of visual excitation. All known visual pigments consist of a protein, opsin, united with a carotenoid, retinene, the aldehyde of vitamin A.

Until recently one major group of animals with conspicuous eyes, the insects continued to resist attempts to study their visual chemistry. It now appears, however, that the photochemistry of insect vision is fundamentally the same as that of the other animals studied.

Occurrence of Retinene and a Photosensitive Pigment

Retinene$_1$ is present in the heads of dark-adapted honeybees.[5] After the tissues are dried by grinding in sodium sulfate, the retinene can be extracted with a polar organic solvent such as acetone. The fact that the retinene can be extracted with acetone or alcohol, but not with petroleum ether, suggests that it is present in the tissues bound to protein. No retinene has been obtained from thoraxes and abdomens. Since the only function known for retinene is that of a chromophore of visual pigments, it is probably present in bees in the compound eyes and ocelli.

As much as 0.2 μg. of retinene per gm. fresh weight of head tissue has been obtained. This corresponds to slightly more than 3×10^{-6} μmole per eye. About $20{,}000 \times 10^{-6}$ μmole of retinene can be extracted from a single retina of dark-adapted cattle; if the sizes of bee and cattle retinas are, respectively, about 2 and 700 sq. mm., it appears that honeybees have about one twentieth the amount of visual pigment per area of retina that cattle have.

Some, if not all, of the bee's retinene is bound to protein to form a photosensitive pigment. Unlike any other known visual pigment, this retinene-protein complex is readily soluble in water without the aid of digitonin or other solubilizing agents. When the heads of dark-adapted bees are ground in a large volume of neutral phosphate buffer at 4° C. under dim red light, most of the retinene protein is brought into solution. However, such an extract is deeply colored by the water-soluble accessory pigments of the ommatidia. These accessory pigments, or "ommachromes," are almost

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entirely light-stable and, when present in such large amounts, they interfere with spectrophotometric analysis of the retinene-protein complex. They can be largely removed by a series of differential precipitations.

After straining through cheese cloth, the initial buffer extract—250 to 300 ml./2000 to 2500 heads—is brought to 45 per cent saturation with ammonium sulfate. A highly colored precipitate forms, but the bulk of the retinene protein remains in solution. The retinene protein is itself largely precipitated from a solution 60 per cent saturated with ammonium sulfate.

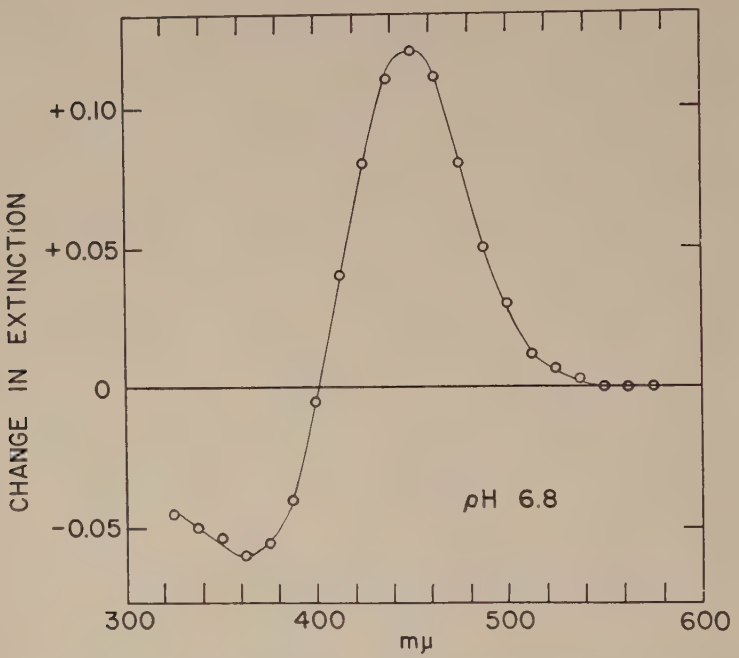

FIGURE 1. Difference spectrum of a photosensitive pigment from the heads of honeybees. An aqueous extract of dark-adapted honeybees was prepared as described in the text. The absorption spectrum was then recorded, and the sample exposed for several minutes to a yellow light. The absorption spectrum was again recorded and subtracted from the first spectrum. The difference is shown. It possesses λ_{max} at about 450 mμ. The true λ_{max} of the photopigment is probably closer to 440 mμ.

This precipitate is redissolved in fresh buffer and dialyzed to remove impurities of low molecular weight. Additional colored contaminants are precipitated in 25 per cent ethanol at −12° C. After further dialysis, an extract of 2000 heads is concentrated in 0.5 to 1.0 ml. at *p*H 6.8 to 7.0. At least one half the retinene originally present is lost during the procedure.

This final solution contains a photosensitive substance with λ_{max} about 440 mμ (FIGURE 1). On bleaching in the light, the extinction decreases in this region of the spectrum and increases around 370 mμ. The increase in extinction at 370 mμ appears to represent the formation of retinene.

Spectral Sensitivity Measurements

For nearly fifty years, K. von Frisch and his co-workers have maintained that the honeybee possesses color vision. The evidence was obtained by training bees to associate food with colored cards or lights of different wave lengths. Von Frisch, in a review of these experiments,[6] concluded that the bee sees four spectral regions as qualitatively different sensations: near ultraviolet (300 to 400 mμ), blue (400 to 480 mμ), blue-green (480 to 500 mμ), and yellow (500 to 650 mμ). Daumer[7] suggested that the primary colors for

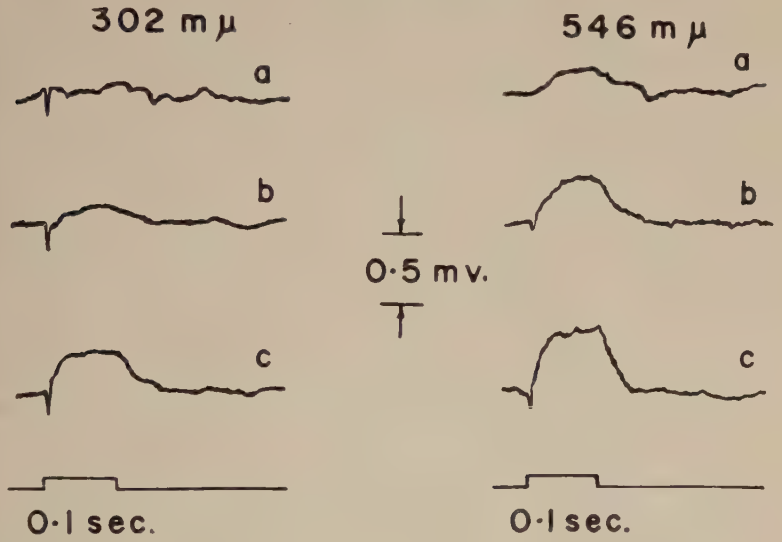

FIGURE 2. The growth of the electrical response of a bee ocellus with increasing intensity of stimulation is illustrated for two wave lengths. There are qualitative differences in the ERG that depend on wave length. The threshold response in the near ultraviolet consists of a brief, positive "on" component; at longer wave lengths, on the other hand, the threshold response is a slow, negative wave. Stimulus durations (0.1 sec.) are indicated below the responses. DC amplification.[8]

the bee are near ultraviolet (300 to 400 mμ), blue (400 to 500 mμ), and yellow (500 to 650 mμ).

To obtain further information, the spectral sensitivities of the ocelli[8] and compound eyes of honeybees have been examined by electrophysiological methods. The results suggest that the 440 mμ photopigment described above is a visual pigment, but only one of several visual pigments present in the bee.

Intact living bees were mounted with tacky wax on a small wooden platform. One compound eye or ocellus was illuminated with brief (one twenty-fifth to one tenth sec.) flashes of light of known spectral content and intensity. The electrical responses to illumination—electroretinograms, or ERGs—were recorded by means of finely tapered stainless steel electrodes, one placed in

the eye and the other at some point in the body indifferent to potential changes occurring in the eye. The responses were amplified with a Grass P4 or P6 amplifier and displayed on an oscilloscope. The lights for stimulation were either the emission lines of a 250-watt mercury arc (302, 313, 334, 365, 405, 436, 491, 546, 578, and 623 mμ) isolated with a pair of quartz monochromators, or various narrow bands of wave lengths (3.3 mμ half-band width) selected with a grating monochromator from the continuous spectrum of a zirconium arc. The relative energies of the various lights were measured

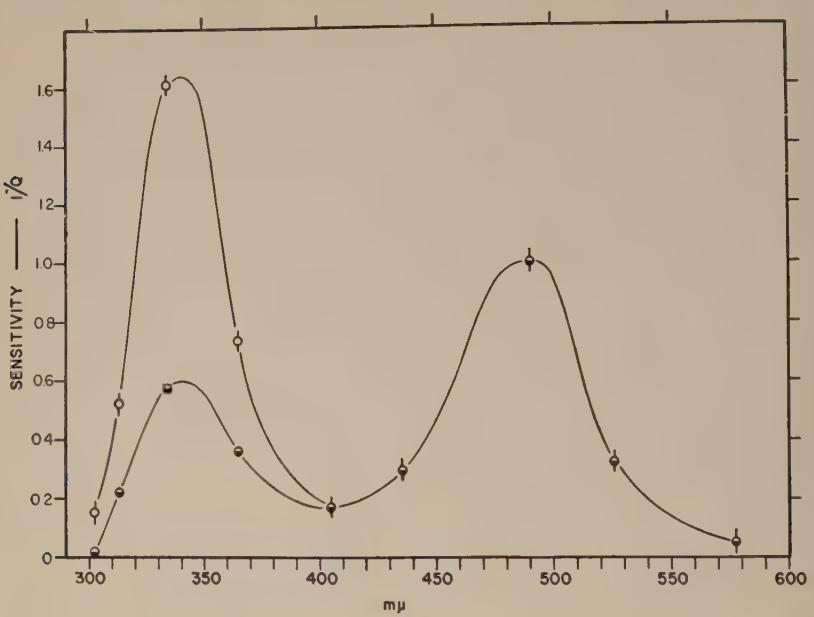

FIGURE 3. The spectral sensitivity of a median ocellus of a worker honeybee. The half-filled circles represent the negative component of the ERG; the open circles, the positive component. Note that the curve has two maxima, at about 490 mμ and 335 to 340 mμ, regardless of whether the curve is based on the positive or negative component of the electroretinogram. Ordinant, reciprocal of the relative number of quanta required to produce a constant electrical response; abscissa, wave length.[8]

with a thermopile and calibrated photocell, and the intensity of each wave length could be varied in a known way by a pair of calibrated photographic wedges.

To make a spectral sensitivity curve, the relative numbers of quanta necessary to produce a retinal response constant in size were determined at each wave length. The reciprocals of these values, each expressed as a fraction of the value at the wave length of maximum sensitivity, provide a measure of sensitivity.

The ocelli of worker honeybees have two types of photoreceptor.[8] A spectral sensitivity curve of the ocellus always has two peaks, one at 490 mμ in the blue-green and one at 335 to 340 mμ in the near ultraviolet. Not only

do these two peaks vary in relative prominence, but the electrical responses of an individual ocellus are qualitatively different in these two regions of the spectrum.

FIGURE 2 shows two series of responses of a dark-adapted median ocellus to lights of increasing intensity. In the near ultraviolet—302 to 365 mμ—the threshold response is a brief, positive deflection at "on." With increasing intensity, the positive component of the ERG grows somewhat, and a

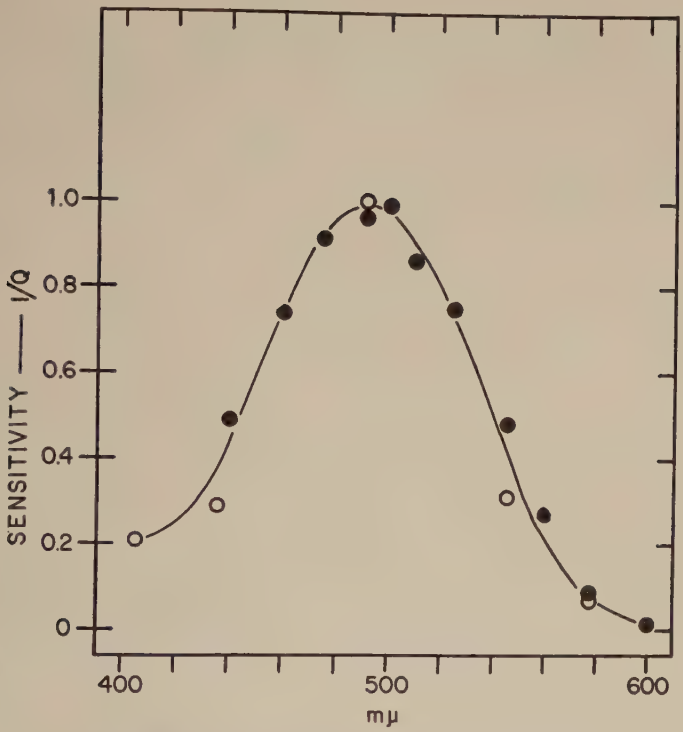

FIGURE 4. Spectral sensitivity of the 490 mμ receptor in the ocellus of the honeybee. The open circles represent the average sensitivities of 4 ocelli stimulated with the mercury lamp; the solid circles, 3 ocelli, with the zirconium lamp. Axes as in FIGURE 3.[8]

slower, negative wave appears. At wave lengths longer than 405 mμ, however, the threshold ERG consists of the slow, negative component alone. Only as the intensity is increased does the positive component appear. At high intensities, responses at both ends of the spectrum are similar in appearance—a brief positive wave is followed by a more prominent, slower negative wave. The transition between the two types of threshold response occurs somewhere between 365 and 405 mμ.

FIGURE 3 is a spectral sensitivity curve of a single median ocellus. Two different criterion responses were used to obtain this curve. The open circles represent the reciprocals of the relative numbers of quanta necessary

to produce a threshold positive "on-spike"; the half-filled circles, the reciprocals of the relative numbers of quanta necessary to produce a 0.3 mμ negative wave. Regardless of which component of the electroretinogram—positive or negative—is used in making the spectral sensitivity curve, two peaks of sensitivity appear, at 490 mμ and at 335 to 340 mμ. FIGURE 4 shows additional measurements of the 490 mμ maximum made with the zirconium lamp.

The compound eyes of honeybees seem to possess several receptors maximally sensitive in different regions of the spectrum. The situation is somewhat more complicated than in the ocellus, however, and a more complete report of the results will appear later. One peak of sensitivity, that in the

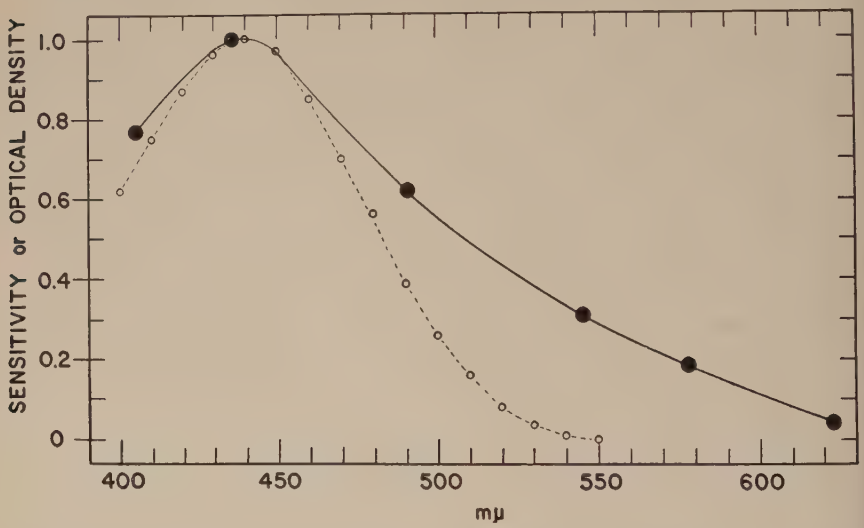

FIGURE 5. Comparison of spectral sensitivity measurements of the compound eye of the drone, represented by the filled circles, solid line, with the absorption spectrum of the 440 mμ photopigment, represented by the open circles, dashed line.

blue-violet, is of special interest, for it will be recalled that the photosensitive pigment described above possesses λ_{max} at about 440 mμ. FIGURE 5 compares an average of 4 spectral sensitivity functions of the compound eyes of dark-adapted drone bees with the absorption spectrum of the photopigment. The latter curve was obtained from a difference spectrum made in the presence of potassium borohydride. The retinene liberated was therefore reduced immediately to vitamin A, with peak of absorption at about 328 mμ and negligible absorption at wave lengths longer than 400 mμ. This difference spectrum, therefore, is a close approximation to the absorption spectrum of the photopigment. The similarity in the positions of the peaks of the two curves in FIGURE 5 suggests that the 440 mμ photopigment is a visual pigment. However, it is not the only visual pigment present. The departure of the two curves at longer wave lengths seems to be caused by a small con-

tribution to the spectral sensitivity function from a visual pigment absorbing maximally in the green.

Summary

The heads of honeybees contain small amounts of retinene$_1$. At least some of this retinene is bound to protein to form a photosensitive pigment with peak of absorption at about 440 mμ.

Spectral sensitivity measurements of the compound eyes and ocelli of honeybees reveal several sensitivity maxima in different regions of the spectrum. One such maximum, which was observed in the compound eye of the drone, is at 440 mμ and is probably based on the 440 mμ photopigment. The ocelli of worker bees possess two types of photoreceptors, which are maximally sensitive at 335 to 340 mμ and at 490 mμ.

The occurrence of several peaks of sensitivity in different regions of the spectrum indicates that the compound eyes and ocelli of honeybees contain several visual pigments, of which the 440 mμ photopigment is but one.

References

1. Wald, G. 1945–1946. The chemical evolution of vision. Harvey Lectures. Ser. **41**: 117.
2. Crescitelli, F. 1958. The natural history of visual pigments. Ann. N. Y. Acad. Sci. **74**(2): 230.
3. Hubbard, R. & R. C. C. St. George. 1957–1958. The rhodopsin system of the squid. J. Gen. Physiol. **41**: 501.
4. Wald, G. & R. Hubbard. 1957. Visual pigment of a decapod crustacean: the lobster. Nature. **180**: 278.
5. Goldsmith, T. H. 1958. The visual system of the honeybee. Proc. Natl. Acad. Sci. **44**: 123.
6. von Frisch, K. 1950. Bees, Their Vision, Chemical Senses, and Language. Cornell Univ. Press. Ithaca, N. Y.
7. Daumer, K. 1956. Reizmetrische Untersuchung des Farbensehens der Bienen. Z. vergleich. Physiol. **38**: 413.
8. Goldsmith, T. H. & P. R. Ruck. 1958. The spectral sensitivities of the dorsal ocelli of cockroaches and honeybees—an electrophysiological study. J. Gen. Physiol. **41**:1171.

THE NATURAL HISTORY OF VISUAL PIGMENTS*

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Many of the visual pigments located in the outer segments of the visual cells of the vertebrate retina can be brought into solution and analyzed spectrophotometrically. Such studies have revealed that these chromoproteins have the same characteristic broad-banded spectrum,[1] and that the photosensitivity, insofar as it has been determined, is not grossly different for the pigments from different animals.[2] One important question pertains to the spectral location of these substances. Is there only a limited number of spectral locations for these compounds, such as the region of 500 mμ for the rhodopsins, 522 mμ for the porphyropsins, and 562 mμ for the iodopsins, or are these substances distributed over broader regions of the spectrum? The answer to this question has biological significance, not only with respect to theories of color vision, but also with regard to the wider problem of the adaptation of organisms to their environment.

Recent work from a number of laboratories, in fact, has revealed the existence in animals of a multiplicity of visual pigments.[3-5] At this laboratory we have been especially interested in the types of visual pigments found in nature and the results of our research, which are summarized in this report, suggest that, within the setting of a system founded on the two retinenes (retinene$_1$ and retinene$_2$), nature has devised a variety of compounds located over broad regions of the spectral scale. It is the purpose of this report to illustrate the nature of this diversity for several vertebrate classes and to suggest, wherever possible, the biological implications. Some of this work has already been published.[6-11] Other work is being presented for the first time. This report constitutes a general statement; details of the experiments and of the results will be published later.

Analytical Methods

The results of an analysis using an extract of the retina of the three-toed sloth (FIGURE 1) will serve to illustrate the general procedures employed in locating the spectral position of a visual pigment within 2 to 3 mμ. The absorption spectrum obtained with an alkaline (pH 8.2 to 8.4) digitonin extract of the retina, outer segments, or whole eyes (when the eyes were very small), was first plotted (curve 1 in FIGURE 1). In the case of the sloth extract, the solution was relatively pure, having the ratio of the density at minimum to density at maximum of 0.24. Assuming that only one photosensitive component was present, which was actually the case for this extract, the wave length for maximal absorption, λ_{max} of the visual pigment from the three-toed sloth is at about 493 mμ. This direct method was

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employed only when a pure extract was available and when the extract contained only one photolabile component. The homogeneity of the extracts was verified by differential bleaching with light at different wave lengths, employing for the initial light exposure a light at the red end of the spectrum.

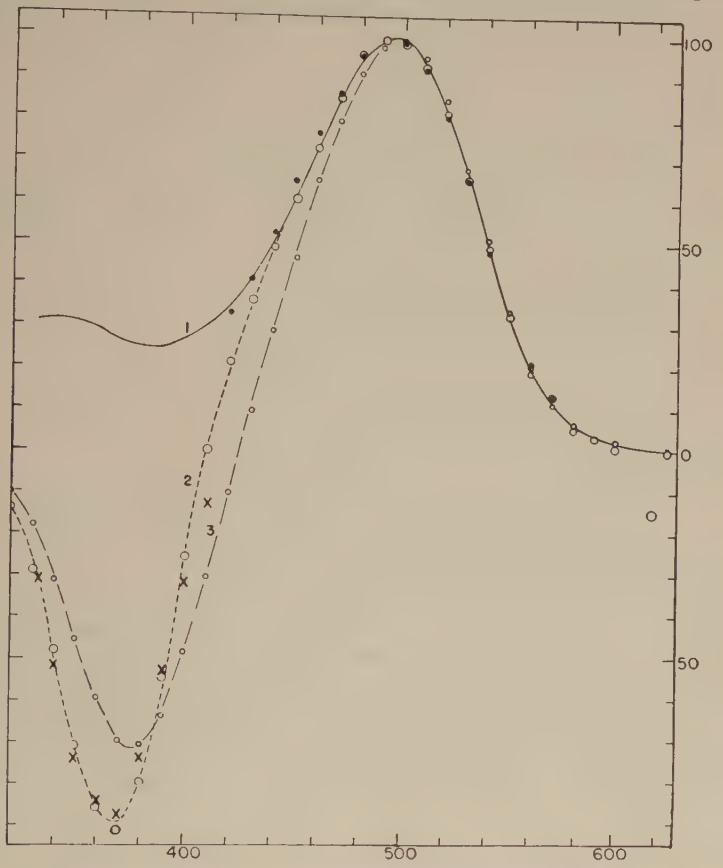

FIGURE 1. Visual pigment of three-toed sloth. Curve 1, the solid line, is the absorption spectrum of unbleached extract. Curve 2, the larger open circles, is the NH_2OH difference spectrum after an exposure to light at 430 mμ. Curve 3, the smaller open circles, is the alkaline difference spectrum after an exposure to light at 606 mμ. The points given by the symbol X (oxime) give the absorption spectrum that resulted when crystalline, all-*trans* retinene$_1$ (donated by Grove Baxter of Distillation Products Industries, Rochester, N. Y.) was allowed to react with NH_2OH in 2 per cent alkaline digitonin solution. The solid dots give the curve constructed from the Dartnall nomogram on the basis of a λ_{max} at 493 mμ. All curves have been scaled so that their maxima coincide.

In the absence of a pure solution, difference spectra were employed. The thermally stable, alkaline extract was exposed to a light of selected wave length. The changes in density at different wave lengths throughout the visible spectrum were plotted as a function of wave length. Curve 3 of FIGURE 1 represents such an alkaline difference spectrum obtained with the

sloth extract. This difference spectrum is characterized by a spectral maximum for loss of density at about 497 mμ, a value slightly greater than the λ_{max} of the spectrum (curve 1) of the unbleached extract. The maximum for the negative portion (the increase in density) is at about 377 mμ in the region characteristic of the retinene$_1$ pigments. The addition of NH_2OH to the extract was helpful in several ways. The difference spectrum obtained after bleaching in the presence of this aldehyde-trapping reagent fits the absorption spectrum of the pure extract over a greater portion of the wave length range than is the case for the alkaline difference spectrum. In the case of the extract from the retina of the three-toed sloth the NH_2OH difference spectrum (curve 2) agrees with the absorption spectrum (curve 1) down to about 440 mμ. The λ_{max} is indicated as well by this NH_2OH difference spectrum as by the absorption spectrum. In addition, the maximum of the negative portion, since it is due to a definite substance—the oxime—is precisely located and is useful in differentiating between retinene$_1$ and retinene$_2$ pigments. In the case of the sloth pigment the λ_{max} for the product (curve 2) was at 366 mμ. This indicates a retinene$_1$ pigment, since crystalline all-*trans* retinene$_1$, when allowed to react with NH_2OH in alkaline digitonin solution, gave a curve (X) that agreed with the negative NH_2OH difference spectrum.

One additional criterion was employed in analysis of these extracts. This was a comparison of the analytical data with the curve constructed by use of the Dartnall nomogram.[1] Dartnall pointed out that the alkaline difference spectra of several visual pigments were all similar in shape when plotted with frequency rather than wave length as the independent variable. On the basis of this fact he devised a nomogram by which the absorption spectrum of any visual pigment may be deduced, once the λ_{max} is known. This last value may be obtained with accuracy, as has been shown, from the NH_2OH difference spectrum. In the case of the sloth extract (FIGURE 1) the solid dots represent the values obtained from the Dartnall nomogram on the assumption of a pigment with λ_{max} at 493 mμ. These points agree well with both the absorption spectrum (curve 1) and with the NH_2OH difference spectrum (curve 2). Occasionally it was found that the reconstructed curve and the absorption curve of a pure extract did not agree exactly. This is explainable on the basis of the presence of more than one photosensitive component in the extract. In several such cases the method of differential bleaching actually did reveal the occurrence of two such pigments.

To summarize, the procedure involved the following steps:

(1) In cases where a pure extract was available the λ_{max} was determined directly, differential bleaching being employed to test the homogeneity of the preparation.

(2) In both pure and impure extracts alkaline difference spectra obtained after selected monochromatic bleachings were useful in describing the long wave-length portion of the absorption spectrum and in locating approximately the wave length of maximum absorption. Providing the extract was stable, the λ_{max} obtained in this way was the same for solutions of varying purity. This fact was pointed out by Crescitelli and Dartnall,[8] and we have

had many occasions to discover the usefulness of this approach in the case of extracts that were impure for various reasons.

(3) Difference spectra obtained in NH_2OH experiments were employed in locating the λ_{max} precisely and in describing the absorption spectrum down to about 430 to 450 mμ. In addition, the position of the product peak permitted a statement as to the nature of the retinene. Retinene$_1$ pigments yielded product maxima in the region of 366 to 368 mμ, while retinene$_2$ chromoproteins resulted in comparable maxima at 385 to 387 mμ.

(4) The absorption spectrum of the unbleached pure extract and the positive portions of the difference spectra, when in agreement with the curve deduced from Dartnall's nomogram, provided reassurance of both the homogeneity of the extract and of the absorption spectrum of the visual pigment.

Evidence of Diversity

The general picture that emerged as a result of the application of the above methods is summarized in FIGURE 2, which shows the wave-length positions for maximum absorption for some of the pigments examined in our labora-

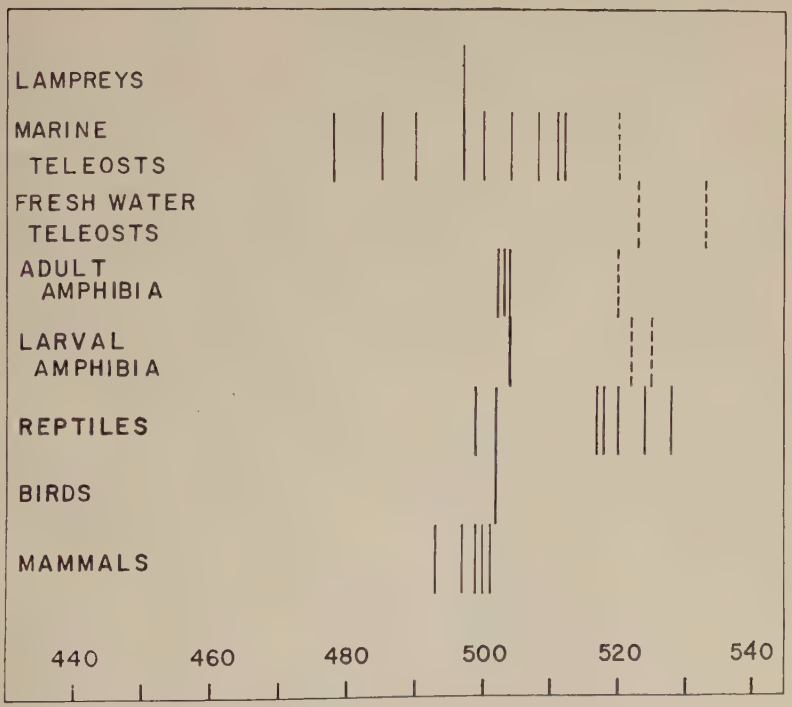

FIGURE 2. The λ_{max} values of visual pigments from various vertebrates. Results are based on NH_2OH difference spectra. The solid line indicates retinene$_1$ pigments, the dotted line, retinene$_2$ pigments. This is an incomplete summary of the work, since the NH_2OH technique has not yet been applied to all visual pigments. It is likely that the diversity is even greater than that shown by these data.

tory. Most of these results are based on analyses by the NH_2OH difference spectrum technique. The results were quite unexpected. Instead of a simple rhodopsin-porphyropsin display, these visual chromoproteins from only this very limited sampling of the animal kingdom range themselves over a considerable portion of the spectrum. The diversity is greater for certain groups of vertebrates than for others. It is especially striking for the marine

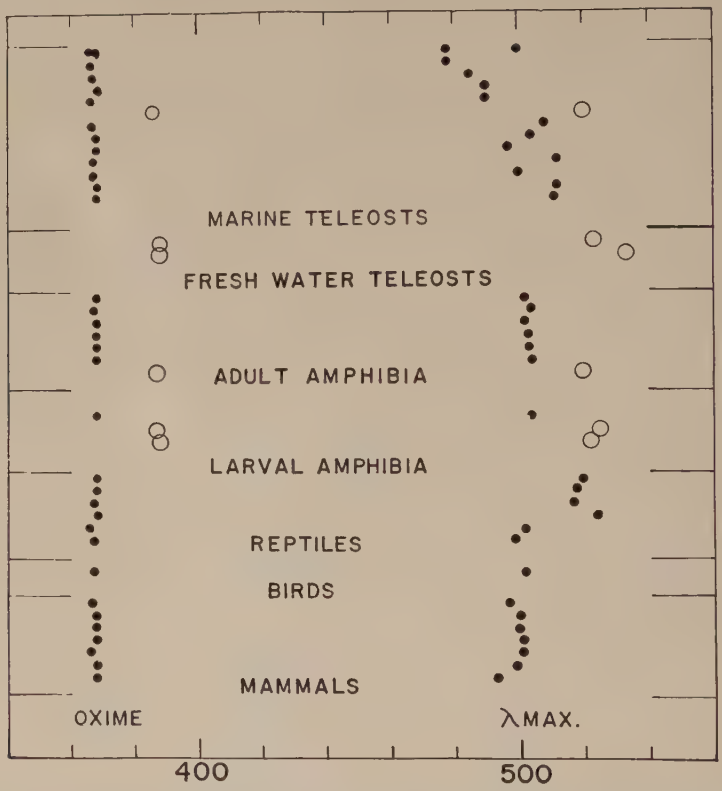

FIGURE 3. Summary chart indicating absorption peaks of visual pigments deduced from the NH_2OH difference spectra and the nature of the retinene. Open circles indicate the retinene$_2$ system and solid dots indicate the retinene$_1$ system. A dual system based on the nature of the chromophore is suggested.

teleosts in which group the work of Munz[12] revealed a spread from 478 mμ to 520 mμ. It is also striking in the reptiles in which a retinene$_1$ system of pigments was noted in the range from 499 mμ to 524 mμ. Diversity has developed in both aquatic and terrestrial vertebrates. Even the mammals revealed some variation, from about 493 mμ to 502 mμ.

In spite of this, Wald's basic idea[13] that the vertebrate visual pigments are associated with two retinenes is supported by the evidence. This is indicated by the data of FIGURE 3 in which the spectral locations of some of the pigments studied by the NH_2OH difference-spectrum procedure are

indicated, along with the positions of the oxime formed after bleaching. It is clear that the pigments, although diverse spectroscopically, yielded but two products: retinene$_1$ (oxime at about 368 mμ) and retinene$_2$ (oxime at about 387 mμ). Nature has devised a mechanism for obtaining considerable spectral adjustment of the visual protein while utilizing only two prosthetic groups. The nature of this mechanism is not known, but the protein portion of the molecule is probably implicated.

Visual Pigments of Specific Vertebrate Groups

Lampreys. Cyclostomes are of interest, not only because of their phylogenetic antiquity, but also because their life cycle involves profound ecologic, morphologic, physiological, and biochemical changes. No direct objective analysis of the visual pigment system of these animals was made until recently when I reported the occurrence of classic rhodopsin in the landlocked Great Lakes sea lamprey, *Petromyzon marinus*, and in the Pacific Coast lamprey, *Entosphenus tridentatus*.[6] This finding was surprising, since Wald[14] had previously concluded, on the basis of vitamin A analyses that the sea lamprey (*P. marinus*, sea-run population) had a preponderance of the porphyropsin system. For my own analyses I re-employed recently transformed downstream migrants of the landlocked sea lamprey and upstream migrants of the Pacific lamprey on their way to the spawning grounds. Wald had used upstream migrants of the sea lamprey. Recently, Wald[15] re-examined this problem with due regard to the stage of metamorphosis of the animals under examination. He first confirmed my finding of rhodopsin in the downstream migrants of the landlocked *P. marinus*. In the upstream migrants of the sea-run form of *P. marinus*, Wald found only porphyropsin, which in this animal possessed a peak at 518 mμ. He interpreted these results as indicating the presence of a dual system of pigments in the sea lamprey, with rhodopsin as the characteristic system of the downstream migrant and of the parasitic phase, and with a change to porphyropsin occurring as the animals approach sexual maturity. Wald's case could have been stronger if he had compared the same ecologic population, as well as the same species. Assuming, however, that the landlocked and sea-run populations behave in the same manner, there is still the larger question of whether all lampreys possess this dual system of pigments and show this metamorphic shift. Since my original publication on this subject, I have examined both downstream and upstream migrants of *E. tridentatus* and have always found the classic rhodopsin system. I have not yet had the opportunity to test lampreys gathered from their nests, so as to assay the pigment of sexually mature adults. It may be that *E. tridentatus* will turn out not to have the metamorphic change suggested by Wald for the sea lamprey. A more thorough general study of lampreys must be made before definitive conclusions about this group of vertebrates can be formulated.

Marine teleosts. Analyses of extracts of the retinas of marine teleosts have demonstrated the presence of a pigment system predominantly of the retinene$_1$ type. This is in accord with the concept proposed by Wald.[13] Spectroscopically, however, these pigments are located, not merely in the

region of 500 mμ, but all the way from about 478 mμ to 525 mμ. Has this array of pigments in these fish any biological significance? Munz,[12] who recently completed a study of 35 species of marine or euryhaline teleosts, has made the following observations.

(1) There is no over-all obvious phylogenetic significance to this array. Taxonomic position is not correlated with the spectroscopic type of visual pigment possessed by a fish. There appears to be some consistent relationship within the family grouping, at least for some teleost families. Thus, the three species, *Oncorhyncus kisutch*, *Oncorhyncus tshawytscha*, and *Salmo gairdnerii*, in the family Salmonidae, all showed the presence of a retinene$_1$ pigment at 507 mμ. In the family Gobiidae, three species, *Clevelandia ios*, *Eucyclogobius newberryi*, and *Gillichthys mirabilis*, revealed another retinene$_1$ system at 512 mμ. One species in this family, *Coryphopterus nicholsii*, was found to be unusual in the possession of a 500$_1$ pigment, in which the subscript indicates the type of chromophore. Two members of the family Embiotocidae, *E. jacksoni* and *Hyperprosopon argenteum*, showed a 506$_1$ pigment; and the cottids, *Cottus* sp. and *Leptocottus armatus*, were found to have a 511$_1$ compound. Genetic factors, probably involving the nature of the protein, may be important in these relationships.

(2) Perhaps the most intriguing suggestion that emerged from recent work on marine teleosts is the idea that these pigments, in some species at least, may be adapted to the quality of the light in the environment. This idea arose directly out of the studies of the pigments in certain deep-sea fishes.

Even before these pigments were known, however, it had been predicted from studies of the spectral composition of the light transmitted by sea water that deep-sea fish might have spectral sensitivity curves with maxima in the blue-green region.[16, 17] Denton and Warren[18] were the first to report the presence in deep-sea fish of retinal photosensitive pigments with absorption maxima in the region of 480 mμ. These substances, called chrysopsins by Denton and Warren, were not studied by the method of extraction, but by means of densitometry applied to the excised retina. At the time that information about Denton and Warren's work reached us, Munz was at sea collecting deep-sea fish for study. He prepared extracts of the retina and his results confirmed the findings of Denton and Warren; he reported the additional fact that these compounds belong to the retinene$_1$ system.[10, 11] Wald *et al.*[19] have also extracted such pigments from the retinas of certain deep-sea fish. They demonstrated that the original photosensitive pigment is regenerated in the dark upon addition to the protein of the neo-b retinene$_1$.

The spectral location of these chromoproteins well below 500 mμ is best suited to the quality of the light transmitted to these oceanic depths. Jerlov[20] reported that the light penetrating to 200 meters has a maximum at 475 mμ, and that at this depth only a narrow spectral band remains. It could be, however, that the retinal pigment is also adapted to the light of biological origin, which at sufficient depths may, in fact, be the only light. The treatises of both Harvey[21] and Johnson[22] suggest that the bioluminescence of deep-sea fish, although variable from species to species, tends to be pre-

dominantly bluish or bluish-green. Recently Kampa and Boden[23] studied the spectral quality of light at various depths of the San Diego trough. They found that the light flashes at 25 and 60 meters were bluish-green in quality, the mean spectral emission curve having a peak at 478 mμ. Kampa and Boden made the point that the intensity of the flashes was often as much as 1000 times the level of the background illumination and that the over-all level of flash illumination actually increased with increasing depths. These investigators also measured the spectral distribution of the light emitted by two crustaceans, *Euphausia pacifica* and *Pyrosoma atlantica*. For *Euphausia* the spectrum was bimodal with a peak at 476 mμ and a lower peak at 515–530 mμ. For *Pyrosoma* a similar curve was recorded with peaks at 482 mμ and 525 mμ. Such a "match" between the color of the emitted light and the absorption spectrum of the photon-capturing substance of the retina could be important in such processes as food capture, mating, and schooling.

These pigments of deep-sea fishes are present in high concentrations within the visual cells. Denton and Warren[24] reported densities between 0.19 and 1.21 in various fishes. This is obviously an adaptation for vision in an environment with low light levels. Such high densities, however, lead to greater thermal breakdown and, it has been assumed, to higher noise levels. The low temperatures of the deep-sea fish environment partly compensate for such an effect. It has also been assumed that a spectral shift to lower wave lengths will reduce the rate of thermal breakdown, leading to a lower noise and thus improving the ability of the eye to detect signals at low light levels.[25] According to this concept the spectral positions of the visual pigments of deep-sea fishes have resulted in a visual system with greater inherent stability than that of other fishes. Recent work, however, has questioned the view that light and heat bleach rhodopsin by the same mechanism and has suggested that only the light effect is specific for the excitatory process.[26] The importance of experiments to determine the *in vivo* noise level and the visual threshold in fishes with a high concentration of a pigment absorbing in these lower spectral regions is suggested by the recent work.

Although the visual pigments of marine teleosts are predominantly of the retinene$_1$ system, a strictly marine fish is occasionally found with a retinene$_2$ type of chromoprotein. Munz has noted this phenomenon, but the experiments are still incomplete. Wald[27] himself admitted an exception to the general rule when he found in the retinas of two species of labrids, *Tautoga onitis* and *Tautogolabrus adspersus*, a great preponderance of vitamin A_2 over vitamin A_1. In our laboratory, however, Munz[28] examined the visual system of the sheepshead, *Pimelometopon pulchrum*, and in this labrid he found only a retinene$_1$ system. The labrids are obviously worthy of further examination.

In the retinal extracts of several marine teleosts, not one but two photosensitive pigments with the chracteristics of visual substances have been noted. The scup, *Stenotomus chrysops*, has been found to contain rhodopsin and another retinene$_1$ photosensitive component with a λ_{max} in the region from 525 to 530 mμ.[29] This second component was found to be present in considerably smaller quantities. From the tautog the same authors[29]

reported the presence of a dual retinene$_2$ system with porphyropsin (522 mμ) and a smaller quantity of a pigment at about 550 mμ. Munz[12] has also noted the occurrence of such dual systems in a number of marine teleosts. Perhaps the most interesting of these double systems was the one found in the deep-sea fish, *Bathylagus wesethi*.[11] This was revealed by the method of differential bleaching, and illustrates clearly the ability of this method to detect heterogeneity. From the hatchet fish, *Argyropelecus affinis*, a relatively pure extract was prepared. The absorption spectrum of this extract and a curve constructed from Dartnall's nomogram, assuming a pigment with λ_{max} at 478 mμ, agreed perfectly down to about 450 mμ. Selective bleaching in the presence of NH_2OH yielded successive difference spectra that were the same following successive exposures to light of 606 mμ, 580 mμ, and 560 mμ. The λ_{max} values of these difference spectra were constant at 478 mμ, and the product peak, at 365 to 367 mμ, was characteristic of a retinene$_1$ system. No evidence of any other component in the extract was obtained. The results obtained with a retinal extract of *Bathylagus* provided a contrast. The extract of the same degree of purity as that from *Argyropelecus* yielded an absorption spectrum that could not be fitted by a curve deduced from the nomogram. The two curves when matched at the peaks showed serious departure at the yellow-orange portion of the spectrum, the absorption spectrum being broader. Selective bleaching in the presence of NH_2OH revealed heterogeneity. An initial exposure to red light (631 mμ) produced a typical difference spectrum with a λ_{max} at 500 mμ and a product peak indicative of retinene$_1$. The positive portion of this difference spectrum was adequately fitted by a curve read off the nomogram, assuming a λ_{max} at 500 mμ. Five successive bleachings with light at 606 mμ, 606 mμ, 580 mμ, 580 mμ, and 560 mμ resulted in a λ_{max} change to 497 mμ, 485 mμ, 480 mμ, 478 mμ, and 478 mμ, during all of which the product peaks remained constant at the retinene$_1$ position. The final difference spectrum was adequately fitted by a nomogram-derived curve with maximum at 478 mμ. These data were interpreted to mean that there are two retinene$_1$ visual pigments in the retina of *Bathylagus*, one at about 500 mμ, the other at about 478 mμ. Munz calculated that the 500$_1$ pigment constituted about 20 to 25 per cent of the total photosensitive system. The idea that such a dual system may have arisen in association with the habit of vertical migration, a characteristic of some of these fish, is merely an intriguing thought. No other deep-sea fish has yet been shown by these methods to yield two visual pigments. A similar experiment with the sheepshead—a labrid—has disclosed another retinene$_1$ system of two pigments, one at about 520 mμ, the other at about 497 mμ.[28]

Duality of another type also occurs in fish, and this involves the possession of both retinene$_1$ and retinene$_2$ systems. Wald[27] is the originator of the idea that euryhaline fishes are characterized by such duality. Bridges[30] put this idea to direct test when he examined extracts of the rainbow trout by means of selective bleaching. In this euryhaline fish Bridges noted the presence of two photosensitive components, one at 533 mμ with retinene$_2$, the other at 507 mμ with retinene$_1$. In our laboratory we also have obtained clear

evidence of such duality in salmon. The interesting question as to whether a change in proportion of the retinene$_1$-retinene$_2$ system accompanies the migrations of fishes such as the salmon and the eel has not yet been tested.* The same kind of lacunae in knowledge exists here as for the lampreys. It should be pointed out, however, that euryhalinity per se is not necessarily associated with such a duality. The mudsucker, *Gillichthys mirabilis*, is able to tolerate great changes in salinity.[32] Extracts of the retina have revealed only one photosensitive component, a retinene$_1$ pigment at 512 mμ.[9] This conclusion was reached from an analysis of the absorption spectra of rather pure extracts and from the results of selective bleaching.

Fresh-water teleosts. The theme of this paper—multiplicity and diversity of visual pigments—arose originally in connection with the visual pigments of fresh-water teleosts. W. Kühne, who actually made the first study of the natural history of visual pigments, saw a difference in color between the retinas of certain fish and the retinas of other animals. Together with H. Sewall, he noted that the retina of the bream, *Abramis brama*, tends to be more violet than purple.[33] He observed that the bream retina is distinctly different in color from the eel retina, which in turn is similar to the frog retina, except that it is more saturated. From the difference in appearance in different spectral lights, he contrived a crude absorption spectrum for the *Abramis* pigment and compared it with a similarly contrived spectrum for the frog pigment. The spectrum of the bream pigment was shown shifted bodily toward longer wave lengths relative to the position of the frog visual purple. Utilizing various portions of the sun's spectrum, these workers also noted the difference in bleaching effectiveness by light of various wave lengths, and the fact that light of different wave lengths acted differently on the frog and bream pigments. In these brilliant studies Kühne anticipated and stimulated much of the modern work on absorption spectra and on selective bleaching of visual pigments.

The difference in the visual pigments of fish and other vertebrates was confirmed spectrophotometrically by Köttgen and Abelsdorff[34] who employed solutions of these compounds in bile. Using difference spectra, these workers made the important observation that, whereas mammals, an owl, and Amphibia have visual pigments at about 500 mμ, the corresponding pigments for the fish were at about 540 mμ. The importance of Köttgen and Abelsdorff's contribution lies, not in precisely locating the positions of the visual systems with which they worked, for in certain aspects recent work has brought out errors, but in the fact that it pointed the way to a discovery of a fundamental natural difference between visual pigments. This discovery was made by Wald. After noting that certain marine fish[35] have visual purple located at 500 mμ and not at 540 mμ, Wald turned to fresh water fish.[36, 37] These were found to be different in that a new visual protein, named porphyropsin because of its color, was found in place of the rhodopsin of marine fishes. Wald observed that a new retinene—retinene$_2$—was

* Carlisle and Denton have noted that the retina of the eel (*Anguilla anguilla*) is golden-colored in the mature stage, whereas it is purple-colored in the immature fish. This information is given in a recent paper by Denton and Walker.[31]

extracted from bleached retina and that, on standing, these retinas yielded a new substance, which turned out to be similar to a new compound extracted from the livers of fresh-water fish by Lederer and Rosanova.[38] This was vitamin A_2. Thus, a new visual system based on retinene$_2$-vitamin A_2 was discovered to be present in the fresh-water fishes. It was soon realized that the fishes examined by Köttgen and Abelsdorff[34] were fresh-water teleosts, which explained their discovery of a pigment in fishes that is distinct from that in mammals, birds, and Amphibia. Wald[37] questioned the significance of the 540 mμ position of the pigments studied by Köttgen and Abelsdorff. He considered their use of difference spectra to be meaningless. To Wald porphyropsin meant, not only the visual pigment of fresh-water fishes, but also the retinene$_2$ pigment with a maximum at 522 ± 2 mμ. Originally Wald held rigidly to this spectroscopic definition, but recently he has broadened the scope of the word porphyropsin to include the visual pigment of the upstream stage of the sea lamprey, which was found to have a λ_{max} at 518 mμ. In any case it is doubtful if the pigments that Wald[37] extracted all had absorption peaks at 522 mμ. For his conclusion Wald utilized only the absorption spectra of the unbleached extracts. These were of varying degrees of purity. As Crescitelli and Dartnall[8] pointed out, the absorption peaks of such extracts are not truly indicative of the pigment peaks. Dartnall[3] noted, for example, that in the case of Wald's pickerel extract, which was the most impure, correction gave a figure of 532 mμ for the pigment maximum. In addition, Wald did not test his extracts for homogeneity, assuming that only one photosensitive component was present. Recent work has demonstrated that such an assumption cannot be made; the homogeneity of an extract must be proved experimentally.

The most recent studies, especially those by Dartnall,[3] have revealed the occurrence of considerable diversity in the visual pigments of fresh-water fishes. In some cases a pigment is found located in the classic position defined by Wald. Such a case is the carp, *Cyprinus carpio*.[8] In other fishes, pigments have been detected at spectral positions significantly different from the 522 mμ location, for example, the tench, *Tinca tinca*. From this fish Dartnall[39] obtained a component with a peak at about 533 mμ. To satisfy myself that a real difference exists in the position of the carp and tench pigments, I recently analyzed, by means of NH_2OH difference spectra, an extract of carp retinas and one of tench retinas sent to me by Dartnall. These two extracts were of about the same degree of purity. Bleaching each of these extracts with red light uncovered the following information. The carp pigment difference spectrum had a maximum for density loss at about 520 mμ and a maximum for density gain at about 387 mμ. The corresponding figures for the tench extract were 533 mμ and 387 mμ. The results clearly indicate that, while both of these pigments belong to the retinene$_2$ system, they are separated spectrally by more than 10 mμ. Dartnall has also reported the occurrence of 533$_2$ pigments in the pike, *Esox lucius*,[39] and in the bleak, *Alburnus lucidus*,[40] but these results have not been confirmed by means of NH_2OH difference spectra. There is also some evidence of two[39] and even of three[40] photosensitive components in retinal extracts from

certain fresh-water fish, but these results have not been accepted by all investigators in the field.

These claims are important enough to merit further investigation. In general, fresh-water fish have been insufficiently examined, and some attention should be devoted to a study of fish from a wider variety of families and from diverse habitats. It would be of interest, for example, to know whether in freshwater fish there is a situation with respect to deep-lake dwellers that is analogous to the deep-sea fish story. One of the most intriguing of recent publications in the visual pigment field is the report of the Japanese investigators[41] who measured the spectra of individual cones of the carp retina by means of microspectrophotometry. After showing that individual rods of the frog and of the carp yielded absorption spectra roughly similar in spectral location to rhodopsin and to porphyropsin, they examined 73 cones, 44 of which bleached to give difference spectra located at 420 to 430 mμ, 490 to 500 mμ, 520 to 540 mμ, 560 to 580 mμ, 620 to 640 mμ, and 670 to 680 mμ. In several cases they were able to record two individual difference spectra from the same cone. This work is certain to arouse considerable discussion.

Amphibia. The amphibian visual pigments extracted by digitonin solution are of two general types, according to whether they are associated with retinene$_1$ or retinene$_2$. In some amphibian species only one of these types was found throughout all developmental stages. In other species pigments of both retinene systems were noted in the extracts. In some of the latter Amphibia a systematic developmental change from the retinene$_2$ to the retinene$_1$ system was observed as an accompaniment to the metamorphic transformation from larva to adult.

With respect to adult Amphibia, the retinas of the adult bullfrog, frog, toad, and tree frog all have the classic rhodopsin. This statement is based on the absorption spectra of relatively pure extracts, on alkaline difference spectra, and on NH_2OH difference spectra. The NH_2OH difference spectra were all characterized by a density loss maximal at 502 to 504 mμ and a density gain maximal at about 368 mμ. Selective bleaching indicated that rhodopsin was the only photosensitive component present in the extracts. It is recognized, however, that some frogs have additional photolabile substances within the rod outer segments. By means of photomicrographic densitometry applied to the isolated fresh retinas of adult frogs, *Rana temporaria*, Denton and Wyllie[42] were able to detect within the so-called green rods a pigment that absorbed strongly in the blue end of the spectrum, bleached readily in blue light and, after bleaching, faded in the blue region. Green rods apparently have, in addition to the photolabile, blue-absorbing substance, a stable material. This was detected in bleached green rods by Hanaoka and Fujimoto,[41] who reported in such rods a spectrum with minimal absorption in the green and increasing absorption on either side of the green. These investigators inferred that this effect might have been due to an interference phenomenon, rather than to the presence of a pigment. With respect to the photolabile pigment of the green rods, Dartnall[3] presented the results of a preliminary analysis of a retinal extract from *Rana esculenta*. After complete bleaching of the visual purple, a further spectral change was

found following exposure to white light, in which density loss was maximal at 430 to 440 mμ. It was suggested that this might have been due to bleaching of the pigment of the green rods. No NH_2OH experiments were reported and the possibility of an isomerizing effect of white light was not ruled out.

The interesting feature of the amphibian rhodopsin system is its location within a very restricted region of the spectrum. This is in contrast to the retinene$_1$ pigments of fish, reptiles, and even of mammals, all of which have an extremely broad representation over the spectral scale. This spectral constancy of amphibian rhodopsin holds true in spite of wide variations in habitat. The same spectroscopic pigment has been noted, for example, in essentially aquatic species such as *Rana catesbeiana* and *Rana aurora draytonii*, and in species such as *Scaphiopus hammondi*, *Bufo boreas halophilus*, *Bufo canorus*, as well as others in which the adults are terrestrial in habit. Again, the rhodopsins from the Yosemite toad, *B. canorus*, and the western toad, *B. boreas halophilus*, are spectroscopically indistinguishable. Yet the Yosemite toad inhabits the higher altitudes of the Sierra Nevada of California, above 6500 feet, where low night and early morning temperatures enforce a degree of diurnal activity not common to other toads. In contrast, the western toad is found at lower altitudes, down to sea level, and is essentially a nocturnal species. This spectroscopic fixity of amphibian rhodopsins suggests a genetic basis for the determination of the spectral properties of these visual pigments.

Not all adult amphibian retinas possess the rhodopsin system. Certain of the more aquatic Amphibia, for instance, *Xenopus laevis* and *Necturus maculosus*, have been shown to have visual pigments whose absorption spectra, compared to the spectrum of classic rhodopsin, are located nearer to the red end of the spectrum. Apparently the pigment of the mud puppy has not been described before, although Wald[43] reported finding only vitamin A_2 in the retinas of these animals. After several unsuccessful attempts, I finally prepared a stable solution of the *Necturus* visual pigment. Three successive bleachings of the extract by light at 700 mμ, 630 mμ, and 500 mμ resulted in typical alkaline difference spectra, which were the same for all three experiments. The maximum density loss in each case was at about 526 mμ, while the maximum density gain was at about 405 mμ. A bleaching experiment in the presence of NH_2OH yielded a difference spectrum with density loss maximal at 522 mμ and density gain maximal at 386 mμ. These facts all point to the presence, in the retinas of this amphibian, of porphyropsin. No evidence was found for the presence of any other photosensitive pigment in the extracts.

The experiments with *Xenopus* furnished similar results. Alkaline difference spectra obtained after bleaching with light at 630 mμ and 420 mμ were characterized by maximum density losses at 522 to 523 mμ and maximum density gains at 396 to 398 mμ. The NH_2OH difference spectrum had comparable figures of 519 to 521 mμ and 388 mμ. In addition, it was possible to extract from bleached extracts of *Xenopus* retinas a pigment that, in chloroform, gave an absorption band at 410 mμ, the position of retinene$_2$ in chloroform. All these data point to the occurrence of a retinene$_2$ system in

Xenopus. The visual pigment system of *Xenopus* was also examined by Dartnall,[44, 45] who first reported the presence of a photosensitive pigment for which the λ_{max} of the alkaline difference spectra was at 520 mμ. Later he amended the account by asserting that *Xenopus* extracts have a mixture of a pigment located at 523 mμ and another at 502 mμ. These were present to the extent of 92 and 8 per cent, respectively. I have not observed this in *Xenopus* extracts, but no special efforts were made to look for this small amount of a second pigment. Wald[46] has reported the occurrence of both vitamin A_2 and vitamin A_1 in a ratio of 95 to 5 in *Xenopus* retinas.

With respect to newts and salamanders, studies of their visual pigments are still in progress, but some preliminary statements are worthy of inclusion in this summary. *Taricha torosa*, the Pacific Coast newt, is a large newt that, after metamorphosis, becomes terrestrial. Adults of this species were collected and extracts were prepared. The alkaline difference spectra were in all respects typical of classic rhodopsin. One NH_2OH difference spectrum showed a loss of pigment maximal at 502 mμ and a product peak at 367 mμ. No special attempt was made to discover the presence of any other pigment in far smaller quantities, but studies of this newt are continuing. In contrast to *Taricha*, adults of the spotted newt, *Diemyctylus viridescens*, are aquatic. Wald[43] pointed out that the retina of this aquatic adult contains only vitamin A_2. I have repeatedly tried to obtain from the retinas of these animals solutions that would permit satisfactory analysis. As yet, the results have not been good. There are indications, however, that a major photosensitive component in the region of 520 mμ was present in the extracts. It was not possible to identify the product peak. The indications are that in these two newts—one terrestrial, the other aquatic—the two visual systems, retinene$_1$ and retinene$_2$, occur respectively.

I have also examined the red eft stage of *D. viridescens*. This is often considered to be a terrestrial subadult stage between the aquatic larva and the aquatic adult.[47] The results were not conclusive, but there was some evidence of the occurrence in the extract of both retinene$_1$ and retinene$_2$ systems. It will be recalled that Wald[43] obtained evidence for the existence of both vitamin A_1 and vitamin A_2 in the retina of the red eft. It is also important to remember that not all populations of this newt pass through the terrestrial, red eft stage. Noble,[48] for example, discovered that *Diemyctylus* from Long Island has no intermediate terrestrial stage; instead, it passes from the larva to the adult through an aquatic stage. Whether this is environmental or genetic is not known, but the study of the visual pigments in these different races or populations might offer a neat quantitative measure of relationships.

Visual Pigments During Metamorphosis

Bufo boreas halophilus. This is an example of an amphibian in which the retinene$_1$ system was found for both the aquatic larval stage and the terrestrial adult. Tadpoles of this toad were collected from a local pond and separated into two groups: (1) animals with tails and no limbs, and (2) animals with tails and with hind limbs in various stages of development.

As it was not possible to remove the retinas from the eyes of these small animals, extracts of the whole eyes were prepared. These extracts were found to have a photosensitive pigment which, on exposure to light, bleached in a typical manner. The difference spectra suggested the presence of a typical rhodopsin, and selective bleaching produced no evidence for the presence of a retinene$_2$ component. No spectroscopic differences were found in the pigments from the two larval groups or between the larval and the adult chromoproteins. In fact, the difference spectra obtained in the experiments with tadpoles and with adult toads could be superimposed. It is possible, of course, that larvae even younger than those employed might have revealed some porphyropsin, but the technical difficulties of such an experiment discouraged attempts in this direction. In any case, it is certain that during the developmental stages when certain other Amphibia exhibited a porphyropsin to rhodopsin change, *Bufo boreas halophilus* showed only a single system. Such a transformation is obviously not essential to the process of emergence from water to land. These results offer confirmation in a toad for the view held by Collins *et al.*,[49] who obtained visual pigment solutions from tadpoles of *Rana temporaria* and *Rana esculenta*. Their solutions were impure and no selective bleachings were carried out, but they concluded that rhodopsin was the only pigment in the extracts.

Hyla regilla. In contrast with the above results, the Pacific tree frog provided direct evidence of a metamorphic shift from a larval retinene$_2$ to an adult retinene$_1$ system. Tadpoles of this species were obtained from the same pond and at the same time as were the toad tadpoles. These larvae were separated into the following four groups: (1) larvae with tails but no limbs, (2) animals with tails and hind limbs, (3) tadpoles with tails and four limbs, and (4) emerging animals with four limbs and having either no tails or tails reduced to stubs. The animals of this fourth group were in fact beginning their terrestrial phase of life. Extracts were made of the whole eyes, and the method of selective bleaching revealed clear evidence of the existence of more than one photosensitive component. This fact is illustrated by the results obtained with the fourth group of animals. Following an initial bleaching with light at 640 mμ, the alkaline difference spectrum showed a maximum loss of density at about 521 mμ and a maximum gain in density at about 389 mμ. A second bleaching with light at 606 mμ led to a difference spectrum with corresponding maxima at 506 mμ and 368 mμ. The density loss resulting from the first bleaching was 0.071; that from the second was 0.076. A final exposure to white light resulted in a further loss of 0.039 with maximum at about 503 mμ. This analysis, although not precise in spotting the spectral positions of these two pigments, is clear enough in demonstrating the presence in the retinal extract of both retinene$_1$ and retinene$_2$ systems. In contrast, the extract prepared from the retinas of adult tree frogs responded to selective bleaching in the manner expected for a homogeneous solution. Only a single photosensitive component with maximum at about 503 mμ was detected. The retinene$_2$ system is lost from the retina, not before emergence of the transformed tadpole, but some time after this event.

Analysis of the other extracts suggests that in the very young tadpole only a retinene$_2$ system was present and that, as development occurred, rhodopsin appeared and increased in quantity, while the original pigment gradually decreased. Bleaching the extract from the first group of larvae with light at 660 mμ resulted in a selective density loss maximal at 524 mμ. Further exposures to light, even to white light, revealed no further change. The animals of the second group responded to selective bleaching by a change of which about 83 per cent was due to a retinene$_2$ system and 17 per cent to rhodopsin. The animals of the third group yielded three good extracts for study. An initial exposure to light at 640 mμ led to an alkaline difference spectrum with maxima for density loss and density gain at 523 mμ and 396 mμ. Further bleaching at 640 mμ yielded a very small change. A final bleach at 606 mμ produced a considerable loss in density with maxima at 506 mμ and 380 mμ. In these extracts the rhodopsin system constituted approximately 25 to 40 per cent of the total photolabile component. The data are clear in pointing to a progressive change during metamorphosis from a retinene$_2$ chromoprotein in the early larva, to a retinene$_1$ system in the adult. This positive finding of a dual system in *Hyla regilla*, in contrast to the negative findings obtained with *Bufo boreas halophilus*, is interesting from several points of view. Both groups of animals were obtained from the same pond at the same time. From the time of hatching they had been reared in the same environment. Presumably their food was identical. The aquatic period of life, the egg and larval stages, is of about the same duration in both these species. Storer[50] gives a figure of between 4 and 6 weeks for *Bufo boreas halophilus* and between 6 and 8 weeks for *Hyla regilla*. In addition, both these species emerge from water at the termination of metamorphosis and, as adults, become essentially terrestrial and nocturnal animals. All of these facts suggest that the possession of a dual system of this kind is an innate characteristic. An investigation of this dual visual pigment system of Amphibia in terms of hormonal effects appears to be a subject worthy of attention.

The bullfrog. It was study of this amphibian that originally led Wald[51] to the thesis of a metamorphic shift of visual pigments. Analysis of the A vitamins produced evidence that both vitamin A_1 and vitamin A_2 were present in the tadpole in proportions differing according to the stage of metamorphosis. An extract of the retinas of tadpoles was prepared by Wald. The pigment was purple in color and the absorption spectrum had a maximum at 516 mμ, "as though porphyropsin mixed with a little rhodopsin were present." Since I was not satisfied with this untested statement concerning the pigment in the bullfrog, about two years ago I prepared solutions from the retinas of bullfrog tadpoles. I found that Wald was correct in all respects in his interpretation of the data. In one experiment, for example, I removed the eyes from 60 tadpoles in various stages of development. The retinas were then obtained, the outer segments were shaken off, and an extract was made of these. The absorption spectrum of this solution was characterized by a density at minimum to density at maximum of 0.69, and by a maximum absorption at 516 mμ. Bleaching this solution with a light

at 670 mμ produced a typical change with maximal density loss at 525 mμ and maximal density gain at 395 mμ. A second bleaching, this time with light at 430 mμ, produced a considerably smaller change with comparable maxima at 506 mμ and 370 mμ. Sufficient experiments comparable to those with the tree frog were also performed to demonstrate that a change from the retinene$_2$ to the retinene$_1$ system occurs in the bullfrog as in *Hyla regilla*. It is perhaps worth mentioning that the length of time spent in metamorphosis is not a determining factor for the presence of the dual pigment system. The bullfrog requires at least 2 years to complete larval development, whereas *Hyla regilla* requires 6 to 8 weeks. It is not likely that the absence of this dual system in *B. boreas halophilus* and in the frogs used by Collins *et al.*[49] is correlated with the short duration of larval development in these amphibians.

Clearly, amphibians as a class have developed a dual system of scotopic visual pigments. Within this general class plan there are variations among different amphibians. Some, such as *B. boreas halophilus*, have a retinene$_1$ system throughout larval and adult life. Others, such as *H. regilla* and *R. catesbeiana*, possess a retinene$_2$ chromoprotein as larvae; during development this is replaced by rhodopsin. In others such as *Xenopus laevis* and *N. maculosus*, the retinene$_2$ system persists to the adult stage and, apparently, although this has not yet been thoroughly investigated, the larval type of pigment persists. This persistence occurs both in *Necturus*, which retains many larval features, and in *Xenopus*, which undergoes considerable metamorphic change. The distribution of these visual proteins in Amphibia is not obviously correlated with taxonomy or with environment. In terms of the ecology of Amphibia, it is difficult to understand what advantage there is in the possession of two visual pigments separated by only 20 mμ. One can speculate that the possession of a retinene$_2$ system by the retina during the critical larval period of cellular reorganization is significant in terms of the metabolic competition for vitamin A between the visual cells and other cells. By utilizing a vitamin A_2 while other cells are employing vitamin A_1, the all-important visual cells are effectively removed from the competition for the general pool of vitamin A and are thus placed in a favorable position to complete their functional development. This is pure speculation, of course, for we know neither what the metabolic requirements of cells for vitamin A are, nor whether cells can utilize vitamin A_2 equally as well as vitamin A_1. In addition, there is the fact that some amphibians do not have a retinene$_2$ pigment. In the present state of our knowledge the only reasonable explanation is that this dual system is a phylogenetic accident in terms of the piscine origin of Amphibia.

Are there any correlations between this metamorphic shift and visual processes? A considerable number of morphologic changes have been reported as occurring in the visual cells of the frog, *R. temporaria*, during metamorphosis.[52] Cones were seen to differentiate first, followed by the appearance and development of the rods. Correlated with these morphologic changes as the tadpoles developed was a shift in spectral sensitivity from a maximum sensitivity in the yellow to a peak in the blue-green. Since this frog was found to have only a retinene$_1$ protein,[49] the above spectral

shift was probably a change from a phototopic to a scotopic system. Evidence of a metamorphic shift in spectral sensitivity from a retinene$_2$ to a retinene$_1$ system was recently obtained in *R. pipiens*.[53]

Reptiles. Until recently, attempts to study the visual pigments of reptiles have yielded poor returns. Kühne,[54] obviously a careful observer, was unable to discover any evidence of a bleachable pigment in the retinas of certain snakes and lizards. He concluded that visual purple was absent from the visual cells of these animals. Köttgen and Abelsdorff[34] were similarly unsuccessful in detecting visual purple in the European tortoise. Similar failures have been recorded in more recent times.[55, 56] On the other hand, Walls[57] reported that he saw a pink, red, or lavender color in the retinas of certain snakes, and that this color was bleached on exposure to light. I have made successful extracts of visual pigment from reptiles.[6] From the rattlesnake, *Crotalus viridis helleri*, rhodopsin with a spectral maximum at 500 mμ was obtained. The NH_2OH difference spectrum also showed an increase in density maximal at about 366 mμ. From the retinas of an alligator, *Alligator mississippiensis*, an extract was prepared; its absorption spectrum had a ratio of density at minimum to density at maximum of 0.28. The absorption maximum of this relatively pure solution was at 500 mμ. Bleaching this solution in the presence of NH_2OH resulted in a loss of density maximal at 499 mμ and a gain in density maximal at 367 mμ. These results were in harmony with the classic view that the scotopic pigment of terrestrial animals is a retinene$_1$ protein with an absorption peak in the neighborhood of 500 mμ. The nature of the visual pigment in the alligator was confirmed by Wald *et al.*,[58] who made the additional interesting observation that, whereas alligator rhodopsin was synthesized following the addition of neo-b retinene to the bleached product, the rate of regeneration, unlike that of frog, cattle, and chicken rhodopsin, was rapid. The rate of dark adaptation of the alligator eye was similarly rapid. Both processes were more similar in kinetics to the corresponding behavior of cone pigments and of cones (frogs, mammals, and birds) than to rod pigments and rods.

An entirely new view of reptilian visual pigments presented itself when I began to study geckos.[6] Stimulated by Denton's finding[59] that the spectral sensitivity curve of *Gekko gekko*, instead of being at 500 mμ, was shifted significantly toward longer wave lengths, I made retinal extracts of several geckos. In these extracts retinene$_1$ photolabile pigments were discovered. The spectral location of these gecko chromoproteins was found to be, not in the region of 500 mμ, but significantly displaced away from 500 mμ toward the red end of the spectrum. For the pigment from the Australian species, *Phyllurus milii*, the location was 524 mμ. After the original work on geckos was published, several other species from various families of the Gekkonoidea were obtained and their visual pigments studied. With the exception of a few species discussed below, all of them were observed to have, not classic rhodopsin, but retinene$_1$ compounds with absorption maxima located in the broad region at 518 mμ to 530 mμ (TABLE 1). The *Phyllurus* pigment is therefore not an isolated quirk of nature, but rather an example of a consistent "lawful" phenomenon.

TABLE 1
VISUAL PIGMENTS OF GECKOS*

	1	2	3	4
Eublepharidae				
Coleonyx variegatus		516		367
Coleonyx variegatus	518		382	
Coleonyx variegatus	518		386	
Sphaerodactylidae				
Sphaerodactylus parkeri	528		378	
Gekkonidae				
a. Diplodactylinae				
Aristelliger praesignis	530			
Phyllurus milii	525		378	
Phyllurus milii	525		380	
Phyllurus milii		524		367
Oedura lesueuri	518		382	
Oedura monilis	521		382	
Oedura monilis		519		368
b. Gekkoninae				
Gehyra mutilata	518		373	
Gehyra variegatus	528		376	
Hemidactylus frenatus		520		368
Hemidactylus turcicus	523		384	
Tarentola mauritanica	528		382	
Tarentola mauritanica	528		376	
Tarentola mauritanica	528		384	

* Column 1 contains the λ_{max} of the alkaline difference spectrum; 2, the λ_{max} of the NH_2OH difference spectrum; 3, the product maximum of the alkaline difference spectrum, and 4, the product maximum of the NH_2OH difference spectrum. The classification followed in this table is from Underwood.[60]

What is the possible biological significance of this unusual visual system of geckos? On this point one can only speculate, but as a basis for such speculation we have the phylogenetic history of the gecko visual cells as expressed in the transmutation theory of Walls.[61, 62] A relevant postulate of this theory is that cones are the ancestral type of visual cell, and that throughout evolution transmutation of cones to rods has taken place in association with the change from a diurnal to a nocturnal habit. The evidence for this view stems from the finding of morphologically intermediate cell types logically dispersed between typical cones and typical rods. Applied to geckos, which are considered to demonstrate this intermediacy to a varied and dynamic degree, the facts are as follows.

(1) Double visual cells are commonly found in the retinas of these lizards.[61, 63]

(2) Although species of geckos have been found that do not have oil droplets[61] in the visual cells, the oil droplet is often a characteristic feature of both the single and the double visual cells.[63] This is thought to be a diagnostic sign of cone origins.

(3) The occurrence of a fovea has been noted in some species of geckos.[63]

(4) The work of Walls[61] suggests that even in those geckos in which transmutation to rods has progressed to a marked degree, there is little summation of rods to bipolar cells. I have a private communication from Katherine Tansley, Institute of Ophthalmology, London, England, which also mentions this small neural summation of the gecko retina.

(5) In contrast to these conelike characteristics, the outer segments of many geckos are enlarged to varying degrees and contain visual pigment in sufficient concentration to be seen or to be analyzed. The gecko retina appears to have gained sensitivity by enlarging the outer segment and forming a large concentration of visual pigment. By refraining from the "trick" of neural summation, geckos have retained a respectable degree of visual acuity. It is of interest to compare some of the morphologic observations with the biochemical results that I have obtained. *Gonatodes fuscus* and *Sphaerodactylus argus* have outer segments that are small and conelike. I have not yet extracted the retinas of *Gonatodes* but, in retinal extracts of *S. argus*, I have found no evidence of photosensitive pigment. *S. parkeri*, although it shows a fovea,[63] has droplet-free visual cells with stout rodlike outer segments. From the alkaline difference spectrum in *S. parkeri* I was able to secure evidence for a retinene$_1$ visual pigment with maximum at 528 mμ. *Aristelliger praesignis* shows a fovea-free retina with visual cells that have colorless oil droplets and outer segments that are significantly enlarged. In one specimen of *Aristelliger* sent me by G. Underwood, University College of the West Indies, Jamaica, West Indies, I was able to detect from the alkaline difference spectrum a photosensitive retinene$_1$ pigment with maximum at about 530 mμ. In *Coleonyx variegatus*, a nonspectacled nocturnal gecko with greatly elongated outer segments that has visual cells without oil droplets, a visual pigment has been noted with absorption maximum at 516 to 519 mμ.

Considered in its entirety the evidence points to the gecko retina as being in a state of intermediacy. Has this retina any physiological evidence of conelike characteristics? Little work has been carried out in this area, but one investigation is worthy of note, namely, the study of Crozier and Wolf[64] in which the critical fusion frequency contour of *S. inaquae* was found to resemble that of a cone response. We can now add the biochemical observation that the visual pigment of geckos, although spectroscopically diverse among different geckos, is intermediate in position between iodopsin and classic rhodopsin, both retinene$_1$ pigments. This intermediacy may be simply a biochemical consequence of an evolutionary change from a cone type of visual pigment to a rod type of visual pigment. Diversity among different species of geckos may be simply the result of the occurrence of independent evolution in different gecko stocks.

In terms of the foregoing ideas one might expect to find in some geckos visual pigments located at or about the classic 500 mμ position and, in fact, a certain amount of evidence for this has already been collected. In one retinal extract of *S. parkeri*, bleaching with light at 631 mμ yielded an alkaline difference spectrum with maximum at about 528 mμ. Density loss at maximum was 0.071. The extract was next exposed to light at 560 mμ.

This caused a selective change with a maximum loss of 0.015 at about 500 mμ. The negative portions of the alkaline difference of both these pigments were typical of retinene$_1$ chromoproteins. From *Hemidactylus frenatus* an extract was prepared that, in the presence of NH_2OH, was initially exposed to light at 640 mμ. The density loss at maximum—520 mμ—was 0.039 and the product formed was characteristic of a retinene$_1$ system. An exposure to white light was then employed; this resulted in a density loss of 0.022 with maximum at about 490 mμ. The product was again indicative of a retinene$_1$ compound. An unusual finding was encountered in an extract from the retinas of two specimens of *Oedura robusta*. No evidence was found for the presence of a typical gecko pigment. Instead, selective bleaching with light at 631 mμ and 606 mμ showed the presence of a component with maximum density loss at about 490 mμ. Perhaps the most interesting of these additional gecko pigments is the one found in retinal extracts of *Oedura monilis*.[65] Evidence for this unusual pigment was obtained by the use of both alkaline and NH_2OH difference spectra. In one experiment without NH_2OH, three successive exposures to light at 660 mμ, 640 mμ, and 606 mμ yielded identical difference spectra with a maximal density loss at 521 mμ and a maximal density gain at about 382 mμ. The final treatment involved an exposure to white light. This resulted in a loss of density maximal at 457 mμ. The NH_2OH experiment was then performed in order to elucidate the nature of this final effect. In the presence of NH_2OH, another sample of the extract was exposed to light at 606 mμ. This resulted in complete bleaching of the main pigment, the difference spectrum showing a loss of pigment maximal at 519 mμ and a product with peak at 368 mμ. The absorption spectrum obtained after this 606 mμ bleach had a distinct upward inflection in the region of 460 mμ. In other words, the spectrum actually revealed the presence of an additional pigment in the extract from which the 519$_1$ pigment had been removed. Final exposure of this extract to white light led to a disappearance of this inflection, the resultant difference spectrum showing a small but definite density loss maximal at about 457 mμ. For *O. monilis*, therefore, clear evidence was obtained for the existence of a blue-sensitive component. All these results concerning the presence of additional pigments in retinal extracts of geckos are preliminary, since the lack of animals has prevented completion of the studies. However, these preliminary examinations suggest a visual pigment system in geckos more diverse than hitherto suspected. It is even possible that these lizards have developed color vision mechanisms.

There is no reason to assume, at present, that transmutation of cones to rods has occurred only in geckos. Walls[61] conceived his transmutation theory as having general biological significance. Although intermediacy of visual cell types and of visual pigments is perhaps most strikingly evident in geckos, other vertebrates have demonstrated similar signs of evolutionary change. Retinene$_1$ pigments with spectral locations in the region 525 mμ to 530 mμ have been reported for several marine fish.[29] From this laboratory, Munz[28] has reported the occurrence of a retinene$_1$ pigment at about 520 mμ in the labrid fish, *Pimelometopon pulchrum* (Ayres). Unlike the

pigments in most geckos thus far studied, these fish pigments were found in the extracts as second components in addition to the classic rhodopsin. Munz, for example, found the ratio of the 497_1 to the 520_1 in *Pimelometopon* to be 85 to 15. It may be of some relevance to point out that Lyall[66] has interpreted the loss of single cones in the trout retina during growth as the result of transmutation into rods. Lyall ventured the opinion that a few of the visual cells he observed might have been in a stage of transition between cones and rods. We have here the extension of transmutation to ontogeny, a concept that strikes still closer to the prerogatives of the duplicity theory.

The visual pigment of the alligator has special interest in this connection. Although this pigment appears to be a classic rhodopsin by the usual criteria of spectroscopic location and retinene composition,[6, 58] it shows an interesting biochemical similarity to iodopsin, which is considered on good evidence to be a cone pigment of several animals.[67] Alligator rhodopsin was found to regenerate, not slowly as does frog rhodopsin, but at a rate comparable to that associated with iodopsin.[58] This was not an *in vitro* artifact because the rate of dark adaptation in the alligator, as measured by means of the electroretinogram, was similarly rapid. The alligator rhodopsin shows a very subtle molecular relationship to iodopsin that is quite independent of the evidence of spectral location. It is logical to speculate that this molecular similarity has significance in terms of the phylogeny of the alligator rods.

The story of the geckos is just beginning, and these animals will be of special interest to visual physiologists. The work of Walls and of Underwood has pointed the way to the unique position of the gecko visual cells. Now that a unique pigment has been found replacing the usual rhodopsin of terrestrial animals, physiologists would do well to re-examine these lizards in the light of the duplicity theory. Crozier and Wolf have pointed the way in this field. Electrophysiological, cytological, biochemical, and behavioral studies could be carried out with profit on these interesting animals. The possibility that geckos possess true color-discriminating mechanisms might well be a subject for experimentation. The geckos are numerous and diverse. The main problem, as I have learned, is to get them alive from the places where they occur to the places where investigators are located.

Birds and mammals. Traditionally, the scotopic pigments of birds and mammals have been thought to belong to the system of classic rhodopsins. To date, the work in our laboratory supports this traditional view, and nothing significantly new or different has appeared. The data in TABLE 2 summarize the essential findings. In addition, a number of points are worthy of brief mention.

(1) Extracts of the retinas of chick embryos 12, 16, 19 and 20 days old have not yet revealed the presence of rhodopsin. This work is still in progress and more data are required before it can be concluded that visual purple appears in the visual cells some time after hatching.

(2) No photosensitive component has yet been detected in the cone-dominated retinas of ground squirrels. Special methods and approaches may be required to obtain information on photopic pigments.

TABLE 2
VISUAL PIGMENTS OF BIRDS AND MAMMALS*

		1	2	3	4
Chicken	*Gallus gallus*	504		379	
Great horned owl	*Bubo virginianus*	504		378	
Great horned owl	*Bubo virginianus*	506		378	
Great horned owl	*Bubo virginianus*	502		378	
Great horned owl	*Bubo virginianus*		502		367
Screech owl	*Otus asio*	505		380	
Gull	*Larus occidentalis*	503		376	
Pelican	*Pelicanus occidentalis*	504		375	
Man	*Homo sapiens*	497		378	
Chimpanzee	*Pan troglodytes*		491		368
Three-toed sloth	*Bradypus tridactylus*	497		377	
Three-toed sloth	*Bradypus tridactylus*		493		366
Sea lion	*Zalophus californicus*	497		377	
Sea lion	*Zalophus californicus*	501		382	
Racoon	*Procyon lotor psora*	502		377	
Opossum	*Didelphis virginiana*		493		366
Opossum	*Didelphis virginiana*		495		366
Merriam kangaroo rat	*Dipodomys merriami*		501		367
Merriam kangaroo rat	*Dipodomys merriami*	504		376	
Desert kangaroo rat	*Dipodomys deserti*		501		366
Mohave kangaroo rat	*Dipodomys mohavensis*		501		366
Mohave kangaroo rat	*Dipodomys mohavensis*	504		376	
Little pocket mouse	*Perognathus longimembris*	503		378	
Little pocket mouse	*Perognathus longimembris*		501		367
Grasshopper mouse	*Onychomys torridus*	502		376	
Grasshopper mouse	*Onychomys torridus*		500		366
Wood rat	*Neotoma lepida*	499		378	
Wood rat	*Neotoma lepida*		498		367

* Column 1 contains the λ_{max} of the alkaline difference spectrum; 2, the λ_{max} of the NH_2OH difference spectrum; 3, the product maximum of the alkaline difference spectrum; and 4, the product maximum of the NH_2OH difference spectrum.

(3) Mammalian visual pigments display some degree of spectral diversity, although they are not as versatile in this respect as are the pigment systems of fish and geckos. Some of the mammalian visual proteins are noteworthy because of the relatively low value of the wave length for maximum absorption. In the three-toed sloth, the opossum, and the chimpanzee the pigment was found to be located below 495 mμ—at about the same position as that of the human visual substance.[68] This location below 500 mμ of many mammalian visual pigments has been noted by others. In the case of several rodents, however, the visual chromoproteins were found in the region slightly above 500 mμ.

Summary

The well-known view that vertebrate rods utilize two discrete colored substances, rhodopsin and porphyropsin, is obviously not supported by the

results presented in this paper. It is impossible to defend such a generalized view of nature in the light of a system of pigments which, for the retinene$_1$ compounds alone, is spread from 478 mμ, in deep-sea fish, to 524 mμ, in geckos. This span of 46 mμ is greater than the separation of the classic rhodopsin system from the classic porphyropsin system. In contrast, the generalized concept of duality in terms of the chromophores, retinene$_1$ and retinene$_2$, is thoroughly supported by the evidence. Nature appears to have achieved simplicity and generality in terms of the carotenoid composition, yet versatility and biological usefulness in terms of the protein moieties of the usual pigments. Again, the proteins emerge as the purveyors of biological specificity.

Such a diversity of substances only emphasizes the difficulties and limitations of present systems of nomenclature for these compounds. Such terms as chrysopsin, rhodopsin, and porphyropsin were adequate for a system of pigments located at just a few discrete and narrow spectral regions. These terms are neither accurate nor useful for a system that is almost continuously variable over a large segment of the visible spectrum. What is the logic in referring to the pigment from a gecko as "gecko rhodopsin," as was done recently?[26] It does not appear red; in fact, the *Phyllurus* pigment at 524 mμ looks exactly like carp porphyropsin. Yet even the term porphyropsin is not sufficiently descriptive or discriminating, since the *Phyllurus* pigment is associated with retinene$_1$ and carp pigment with retinene$_2$. It is my opinion that a system that gives the spectral location and the nature of the chromophore, along with the name of the animal in parentheses, is simple, accurate, and sufficiently descriptive. In such a system, for example, the pigment from the gecko *Hemidactylus frenatus* would be designated as 520$_1$ (*H. frenatus*). This system will work for a species having more than one pigment, as in the case of the deep-sea fish, *Bathylagus wesethi*. More complicated systems conveying more information could be devised, but at this stage of development a practical system should be simple as well as accurately descriptive.

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References

1. Dartnall, H. J. A. 1953. Brit. Med. Bull. **9**: 24.
2. Dartnall, H. J. A. 1957. Paper No. 3. Symposium on Visual Problems of Colour. Natl. Phys. Lab. Teddington, Middlesex, England.
3. Dartnall, H. J. A. 1957. The Visual Pigments. Methuen. London, England.
4. Morton, R. A. & G. A. J. Pitt. 1957. Paper No. 2. Symposium on Visual Problems of Colour. Natl. Phys. Lab. Teddington, Middlesex, England.
5. Morton, R. A. & G. A. J. Pitt. 1957. Progr. Chem. Org. Natural Products. **14**: 244.
6. Crescitelli, F. 1956. J. Gen. Physiol. **39**: 423.
7. Crescitelli, F. 1956. J. Gen. Physiol. **40**: 217.
8. Crescitelli, F. & H. J. A. Dartnall. 1954. J. Physiol. **125**: 607.
9. Munz, F. 1956. J. Gen. Physiol. **40**: 233.
10. Munz, F. 1957. Science. **125**: 1142.
11. Munz, F. 1958. J. Physiol. **140**: 220.
12. Munz, F. 1958. Ph. D. Dissertation. Univ. Calif. Los Angeles, Calif.
13. Wald, G. 1942. Visual mechanisms. Biol. Symposia. **7.** Cattell. Lancaster, Pa.
14. Wald, G. 1942. J. Gen. Physiol. **25**: 331.
15. Wald, G. 1957. J. Gen. Physiol. **40**: 901.
16. Clarke, G. L. 1936. Ecology. : 452.
17. Marshall, N. B. 1954. Aspects of Deep Sea Biology. Hutchinson's. London, England.
18. Denton, E. J. & F. J. Warren. 1956. Nature. **178**: 1059.
19. Wald, G., P. K. Brown & P. S. Brown. 1957. Nature. **180**: 969.
20. Jerlov, N. G. 1951. Reports of the Swedish Deep-Sea Expedition 1947–48. III. Phys. & Chem. Fasc. **1**: 1. Elanders. Göteborg, Sweden.
21. Harvey, E. N. 1952. Bioluminescence. Academic Press. New York, N. Y.
22. Johnson, F. H. 1955. The Luminescence of Biological Systems. Am. Assoc. Advance. Sci.
23. Kampa, E. & B. P. Boden. 1957. Deep-Sea Research. **4**: 73.
24. Denton, E. J. & F. J. Warren. 1957. J. Marine Biol. Assoc. U. K. **36**: 651.
25. de Vries, H. 1949. Documenta Ophthalmologica. **3**: 137.
26. Hubbard, R. & R. C. C. St. George. 1958. J. Gen. Physiol. **41**: 501.
27. Wald, G. 1941. J. Gen. Physiol. **25**: 235.
28. Munz, F. W. 1958. Nature. In press.
29. Wald, G., P. K. Brown & P. H. Smith. 1954. Federation Proc. **13**: 316.
30. Bridges, C. D. B. 1956. J. Physiol. **134**: 620.
31. Denton, E. J. & M. A. Walker. 1958. Proc. Roy. Soc. London. **B148**: 257.
32. Weisel, G. F. 1948. Physiol. Zool. **21**: 40.
33. Kühne, W. & H. Sewall. 1880. Untersuch. Physiol. Inst. Univ. Heidelberg. **3**: 221.
34. Köttgen, E. & G. Abelsdorff. 1896. Z. Psychol. Physiol. Sinnesorg. **12**: 161.
35. Wald, G. 1936. J. Gen. Physiol. **20**: 45.
36. Wald, G. 1937. Nature. **139**: 1017.
37. Wald, G. 1939. J. Gen. Physiol. **22**: 775.
38. Lederer, E. & W. Rosanova. 1937. Biochimica. **2**: 293.
39. Dartnall, H. J. A. 1952. J. Physiol. **116**: 257.
40. Dartnall, H. J. A. 1955. J. Physiol. **128**: 131.
41. Hanaoka, T. & K. Fujimoto. 1957. Japan. J. Physiol. **17**: 276.
42. Denton, E. J. & J. H. Wyllie. 1955. J. Physiol. **127**: 81.
43. Wald, G. 1952. Modern Trends in Physiology and Biochemistry. Academic Press. New York, N. Y.
44. Dartnall, H. J. A. 1954. J. Physiol. **125**: 25.
45. Dartnall, H. J. A. 1956. J. Physiol. **134**: 327.
46. Wald, G. 1955. Nature. **175**: 390.
47. Gage, S. H. 1891. Am. Naturalist. **25**: 1084.
48. Noble, G. K. 1926. Am. Museum Novitates. **No. 228.**

49. Collins, F. D., R. M. Love & R. A. Morton. 1953. Biochem. J. **53**: 632.
50. Storer, T. I. 1925. A Synopsis of the Amphibia of Calif. Univ. Calif. Press. Berkeley, Calif.
51. Wald, G. 1946. Harvey Lectures. Ser. **46**: 117.
52. Birukow, G. 1949. Z. vergleich. Physiol. **31**: 322.
53. Kennedy, D. A. 1957. J. Cellular Comp. Physiol. **50**: 155.
54. Kühne, W. 1877. Untersuch. Physiol. Inst. Univ. Heidelberg. **1**: 15.
55. Bliss, A. F. 1946. J. Gen. Physiol. **29**: 277.
56. Wald, G., P. K. Brown & P. H. Smith. 1953. Science. **118**: 505.
57. Walls, G. L. 1932. Science. **75**: 467.
58. Wald, G., P. K. Brown & D. Kennedy. 1957. J. Gen. Physiol. **40**: 703.
59. Denton, E. J. 1956. J. Gen. Physiol. **40**: 201.
60. Underwood, G. 1954. Proc. Zool. Soc. London. **124**: 469.
61. Walls, G. L. 1934. Am. J. Ophthalmol. Ser. 3. **17**: 892.
62. Walls, G. L. 1942. Visual mechanisms. Biol. Symposia. **7.** Cattell. Lancaster, Pa.
63. Underwood, G. 1951. Nature. **167**: 183.
64. Crozier, W. J. & E. Wolf. 1939. J. Gen. Physiol. **22**: 555.
65. Crescitelli, F. 1958. Science. **127**: 1442.
66. Lyall, A. H. 1957. Quart. J. Microscop. Sci. **98**(2): 189.
67. Wald, G., P. K. Brown & P. H. Smith. 1955. J. Gen. Physiol. **38**: 623.
68. Wald, G. & P. K. Brown. 1958. Science. **127**: 222.

NUTRITIONAL NIGHT BLINDNESS*

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Night blindness appears to be the earliest symptom of vitamin A deficiency. The experimental study of night blindness in human subjects has revealed two basic inconsistencies.

(1) On assuming a vitamin A-deficient diet, some subjects begin immediately to go night-blind, whereas others show no effects, visual or otherwise, for many months.

(2) Of the human subjects who do develop various degrees of night blindness on a vitamin A-free diet, some can be cured within a few hours by administration of a large dose of vitamin A, whereas others, although they show some immediate improvement, require months of high vitamin A feeding to return to normal (Wald *et al.*, 1938; Wald and Steven, 1939; Hecht and Mandelbaum, 1940; Wald *et al.*, 1942; Hume and Krebs, 1949).

The first of these inconsistencies has been partially clarified. It is now well established that vitamin A is stored in the liver and transported to the eye via the blood. Much evidence has accumulated to indicate that when the level of vitamin A in the blood drops below a critical point, the eye does not receive sufficient vitamin A and night blindness results (Hume and Krebs, 1949; Bodansky *et al.*, 1941). Moore (1953) and others have shown that human individuals and populations vary greatly in the amounts of vitamin A stored in the liver, and have suggested that susceptibility to night blindness is dependent on this factor.

Animal experiments confirm this view. Lewis *et al.* (1942) and Brenner *et al.* (1942) have shown that rats placed on a deficient diet initially deplete their liver stores while maintaining maximal blood levels of vitamin A. Later the blood levels fall and, still later, the eye levels.

Concerning the second inconsistency, that of the slow and fast cures of night blindness, little progress has been made. Some years ago Tansley (1933) and Johnson (1943) found that in severe vitamin A deficiency in rats the retina deteriorates histologically, beginning with the outer segments of the rods and eventually progressing to the other retinal layers. It has now been established that rhodopsin must play an important structural role in the rod outer segment, since it constitutes a large percentage of the rod proteins. In frogs, for example, 60 per cent of the rod protein is rhodopsin. It may be, therefore, that vitamin A deficiency results in some destruction of the protein opsin. Opsin has been the forgotten component in vitamin A deficiency. It, too, is a precursor of rhodopsin, along with vitamin A and retinene, and any disturbance to opsin would have profound visual effects. It may well be that the disappearance of opsin also leads to such structural

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deteriorations of the rod as Tansley and Johnson have described (Wald, 1955).

With a view to clarifying these relationships, we have attempted to follow the entire course of vitamin A deficiency, measuring as many factors—biochemical, physiological, and histological—as we could.

Male albino rats between 22 and 24 days old were divided into two groups, one of which received the standard U.S.P. vitamin A-deficient diet, the other a complete diet. FIGURE 1 shows biochemical measurements from one such

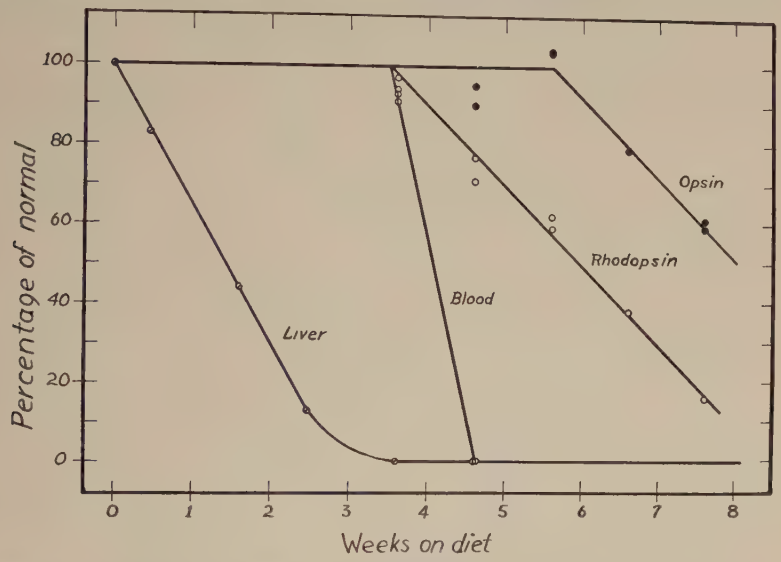

FIGURE 1. Biochemical changes in a group of white rats on a vitamin A-deficient diet. The animals were between 22 and 24 days old when the diet was begun. The liver vitamin A began at once to fall and, within 3 weeks, had reached low values. Then, within a week, the blood level fell from normal to zero. With this, the rhodopsin content of the retina declined, marking the onset of night blindness. Later, the opsin also declined, marking the beginning of the histological deterioration of the retina.

experiment in which we have followed the vitamin A content of the liver, the blood vitamin A, and the concentration of rhodopsin and opsin in the retina. Rhodopsin was measured by direct extraction from the retina; opsin was measured by the retina's ability to regenerate rhodopsin on incubation of bleached retinas with neo-b retinene. The results are expressed throughout as percentages of average control values, except for the liver vitamin A, which is rendered as percentages of the stores initially present.

As soon as the rats were placed on the vitamin A-deficient diet, the vitamin A of the liver began to fall, at first linearly, the average loss being about 2 to 2.5 μg. per day. By the middle of the fourth week the liver was wholly depleted. Thus far the blood vitamin A had remained normal. Now it, too, began to fall rapidly and, within a few days, had reached very low values. At this point, the rhodopsin content of the eye began to decline, marking the beginning of night blindness.

During this period the rats continued to gain weight and appeared normal in all respects. For a time longer, although with little or no measurable vitamin A in the blood, they continued to carry on normally, gained slightly in weight, and displayed no symptoms except the rise of visual threshold.

Then, in the fifth and sixth weeks, the weight leveled off, the rhodopsin continued to fall, and the log threshold to rise, both linearly and regularly. Curiously enough, for an interval of about two weeks, the opsin level remained normal, although the rhodopsin was declining. During this period the rats had more opsin in the rods than there was vitamin A to combine with.

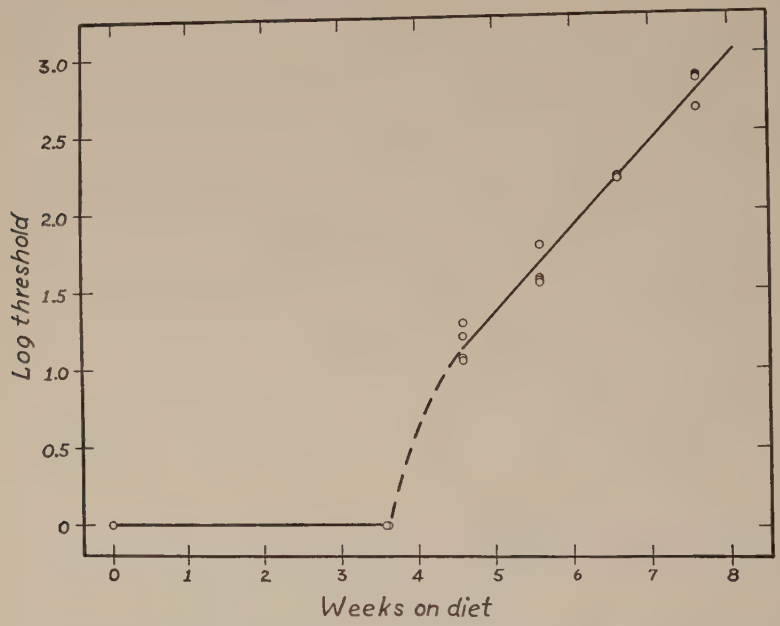

FIGURE 2. Development of night blindness as a result of the vitamin A-deficient diet. Each point shows the logarithm of the ERG threshold of a single animal, the normal threshold being set arbitrarily at 1 (log threshold = 0). After a rapid initial rise, the log threshold rises linearly with time on the diet.

By the beginning of the seventh week, the opsin, too, began to decline and, thereafter, it also fell in linear fashion. At this time the classic signs of the deficiency appeared. The rats lost weight rapidly and xerophthalmia, xerosis, and keratomalacia were observed in the last weeks of the deficiency. The eyes were often surrounded by a reddened area and became encrusted with dried exudate. The animals' coats were often rough and disarranged, and their gait was unsteady. Several of the animals breathed with difficulty and, by the end of the eighth week, all the animals not sacrificed in earlier experiments had died.

FIGURE 2 shows the logarithmic rise of visual threshold with weeks on the diet, as measured by the electroretinogram. The normal threshold is arbitrarily given the value 1; hence the normal log threshold is zero.

For these measurements the animals were adapted to the dark overnight and anesthetized lightly with Nembutal. The ERG was recorded by means of two cotton wick electrodes moistened with Ringer's solution. The active electrode was placed on the side of the cornea and the indifferent electrode on a shaved area on the cheek. The electrodes were connected to a capacity-coupled Grass P4 preamplifier used with a Dumont oscilloscope. Various intensities of test light were obtained by means of neutral filters and circular wedges. Flashes of one fiftieth of a second were employed. To determine the threshold, the test light was set below the threshold and flashed every

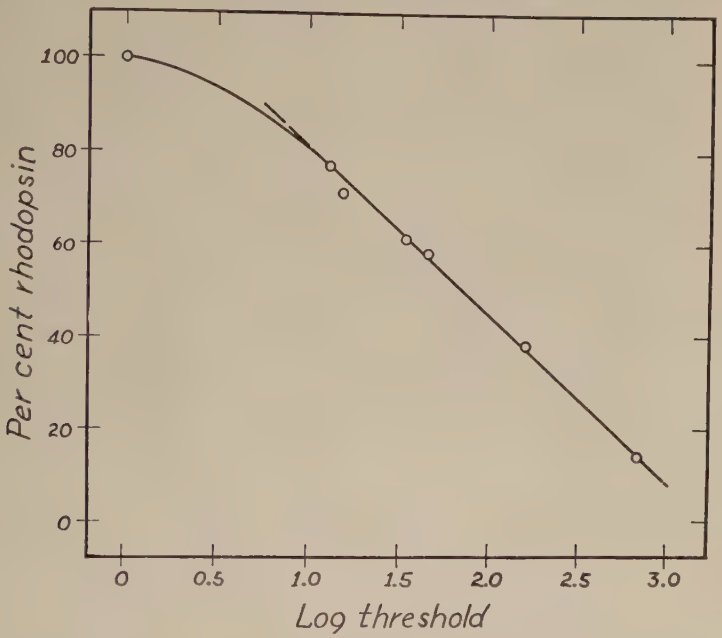

FIGURE 3. Rise of the ERG threshold with decline in rhodopsin content in the retinas of vitamin A-deficient rats. The normal log threshold is set at 0. Rhodopsin is expressed as a percentage of that found in control animals of the same age. Over most of its course, the log threshold rises linearly as rhodopsin falls, following a disproportionately large rise of the threshold with the first decline of rhodopsin.

few seconds, each flash having an increased light intensity, until a response was detected on the oscilloscope trace. This was repeated until constant threshold readings were obtained.

The threshold remained normal through the fourth week, the period in which vitamin A was leaving the liver and blood. Then, with the first decline of rhodopsin, it rose rapidly. Except for a more rapid initial rise, the log threshold rose in a linear fashion through the eighth week. This linear rise of log threshold with time is similar to the linear rise of log threshold observed earlier in human vitamin A-deficient subjects (Wald *et al.*, 1938; Hecht and Mandelbaum, 1940).

FIGURE 3 shows the relationship between rhodopsin concentration and the

log threshold of the dark-adapted eye. Except for a somewhat more rapid initial effect, the rise of log threshold or fall of log sensitivity is linear with the decline of rhodopsin concentration. Here, then, as in human visual

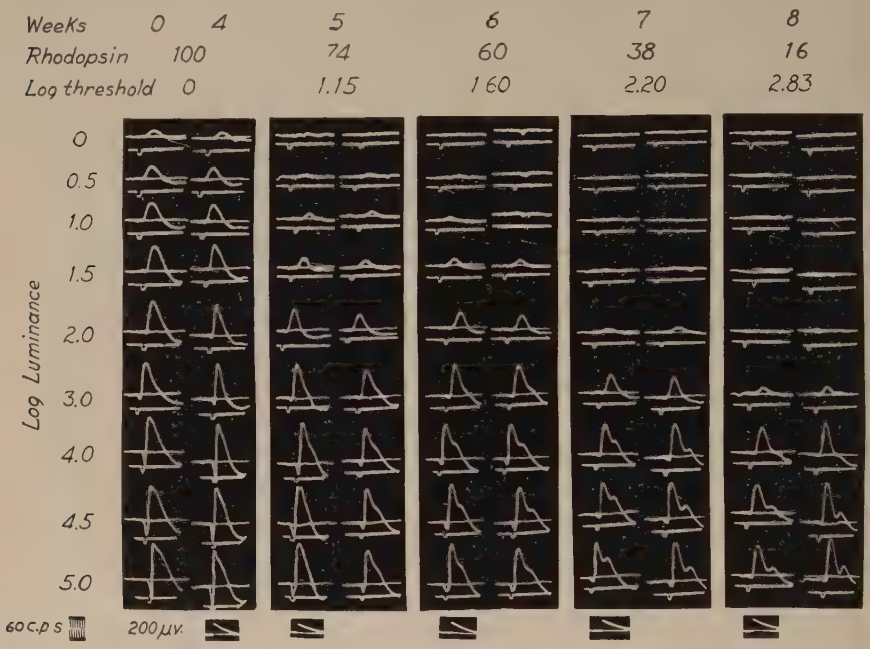

FIGURE 4. Effects of vitamin A deficiency on the electroretinogram (ERG). The top three lines show the number of weeks on the deficient diet, the rhodopsin content of the retinas as percentages of normal, and the logarithm of the luminance of light needed to evoke a just perceptible ERG, the average threshold of normal animals being set arbitrarily at 1 (log threshold = 0). ERGs are shown in response to a range of luminance of 5 log units, that is, 1 to 100,000. Below each ERG, a marker shows the $\frac{1}{50}$-sec. flash. The small rectangles at the bottom show a trace of the 60 cycle AC to indicate the time scale, and weekly calibrations of the oscillographic response to a pulse of 200 μv. The first two vertical rows of ERGs show responses from a rat about to begin the diet and, from another, after 4 weeks, when the vision is still normal. Thereafter records are shown from a pair of animals each week. As the rhodopsin declines, the ERG threshold rises (night blindness), and the ERG displays characteristic changes: (1) the b-wave at each level of luminance declines; (2) the a-wave declines still more rapidly; and (3) an inflection on the downward sweep of the b-wave is increasingly delayed until it appears as a separate positive wave.

adaptation, the log sensitivity varies over most of its range in parallel with the concentration of visual pigment.

Characteristic changes are noted in the form of the ERG as the deficiency progresses. FIGURE 4 shows electroretinograms measured over a range of 5 logarithmic units (1 to 100,000) of luminance. For the first four weeks of the diet, the ERG did not change appreciably. The figure begins, therefore, with the ERGs obtained from a normal animal and from one on the diet for four weeks. For each week thereafter, the ERG was recorded in pairs of animals.

The data at the top of this figure indicate: (1) the week in which the ERG was recorded; (2) the average rhodopsin concentration of the two animals; and (3) the average rise of log threshold in these animals.

In the fourth week, the ERG was normal. With the first decline of rhodopsin in the fifth week, the visual threshold began to rise and continued to rise throughout the course of the experiment. Through the succeeding weeks other changes were noted. As the threshold continued to rise and the rhodopsin to decline, the b-wave at low and middle luminances (that is, up to log luminance 3) was greatly reduced in size. At the higher luminances (that is, log luminances 4 and 5) the b-wave remained nearly normal in size and latency, even when the rhodopsin had declined to only 16 per cent of normal, as in the eighth week.

The a-wave, on the other hand, declined rapidly at all luminances. This change is most striking at the high luminances. At log luminance 5, for example, the normal animals displayed a negative a-wave comparable in size to the b-wave. In the fifth week of the diet, which marked the beginning of night blindness, the a-wave was drastically reduced and, by the eighth week, the a-wave was barely perceptible even at the highest luminances.

The ERG at the highest luminances displays a delayed positive component which, in normal animals, appears only as a small inflection at the top of the b-wave. As the deficiency progressed, this was increasingly delayed; it finally emerged as a separate wave. This wave, the source of which has not been determined, does not appear to represent an off-effect.

All these changes, biochemical and physiological, are reversed by the administration of vitamin A. FIGURE 5 shows the course of recovery in one night-blind animal in the seventh week of deficiency. The animal was injected intraperitoneally with a large dose (1 mg.) of all-*trans* vitamin A dissolved in oil. At the top of the figure are indicated the number of hours after injection and the log threshold.

The threshold fell slowly and reached the normal level within 64 hours. During this period the ERG retraced in reverse all the changes that had occurred during the development of the deficiency. The b-wave at moderate luminances increased in size, the delayed positive component was reassimilated into the main body of the b-wave, and the a-wave increased greatly in size.

FIGURE 6 shows the recovery of visual threshold in several such animals injected with all-*trans* vitamin A and, in one case, with neo-b vitamin A, the hindered *cis* form (11-*cis*), which serves as the precursor of rhodopsin.

On the left of the figure are shown the results of intraperitoneal injection with large doses, 360 to 920 gamma, of all-*trans* vitamin A. The log threshold falls linearly in each case and reaches normal values within 30 to 60 hours, depending on the original degree of deficiency.

On the right is the result of an experiment in which two animals, comparably deficient, were injected, one with all-*trans* vitamin A, the other with neo-b vitamin A. The rat injected with all-*trans* vitamin A recovered, as usual, in a linear fashion. The rat injected with neo-b vitamin A displayed an initial delay in recovery. We have examined the blood of this animal and the livers of this and other animals injected with neo-b vitamin A, and

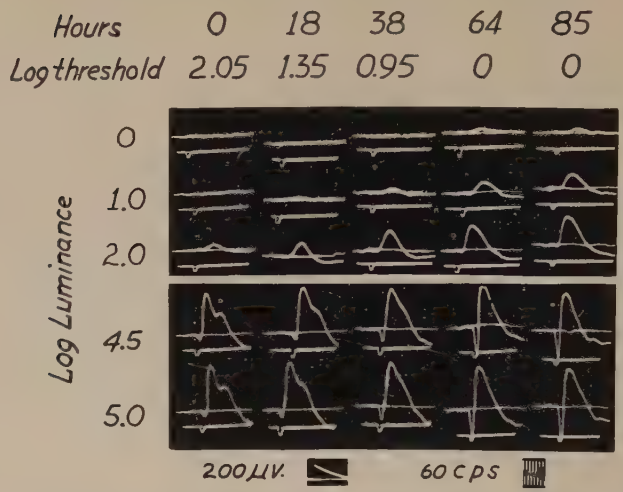

FIGURE 5. Recovery from night blindness on administration of vitamin A. Following intraperitoneal injection of a large dose of vitamin A, the ERG threshold returns within 64 hours to normal (log threshold = 0). During this interval the ERG retraces in reverse all the changes that had accompanied the development of night blindness.

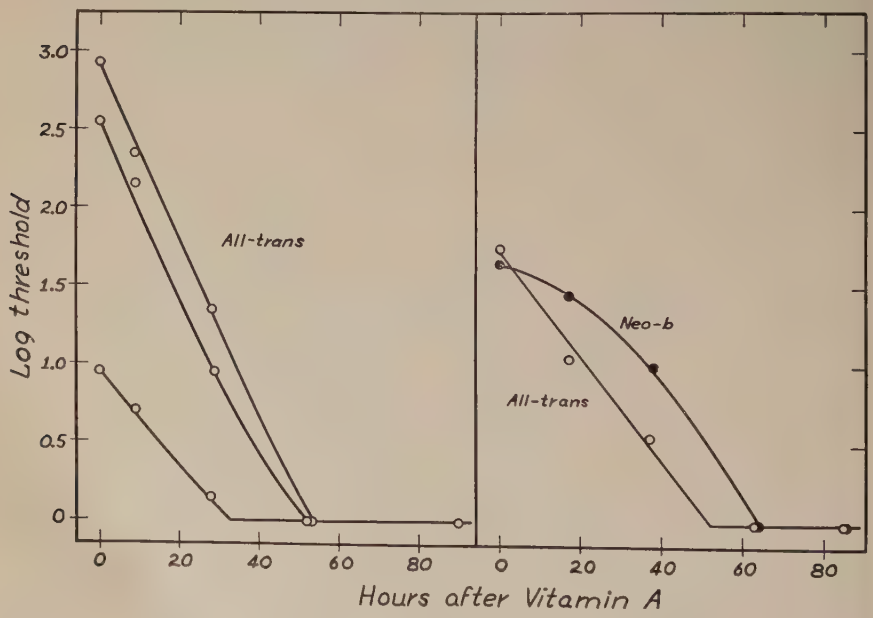

FIGURE 6. Recoveries from night blindness on administration of vitamin A. Log ERG thresholds measured at various times after intraperitoneal injection of large doses of all-*trans* vitamin A (open circles), or neo-b (11-*cis*) vitamin A (solid circles). After injection of the all-*trans* isomer the log threshold falls almost linearly to the normal level. With neo-b vitamin A, however, there is a lag in recovery apparently caused by the necessity to isomerize this configuration primarily to all-*trans* before it can be used by the eye.

have never detected the neo-b isomer in either blood or liver. Furthermore, animals injected with neo-b vitamin A were found to store considerably lower fractions in the liver than do the rats injected with all-*trans* vitamin A. For comparably large amounts of vitamin A injected, about 10 per cent of the all-*trans* isomer was recovered from the liver, but at most 4 per cent of neo-b vitamin A.

Our interpretation of these experiments is that the body must isomerize neo-b vitamin A to the all-*trans* form before it can store it or use it in the eye. This in turn is isomerized to the neo-b isomer, probably in the eye itself.

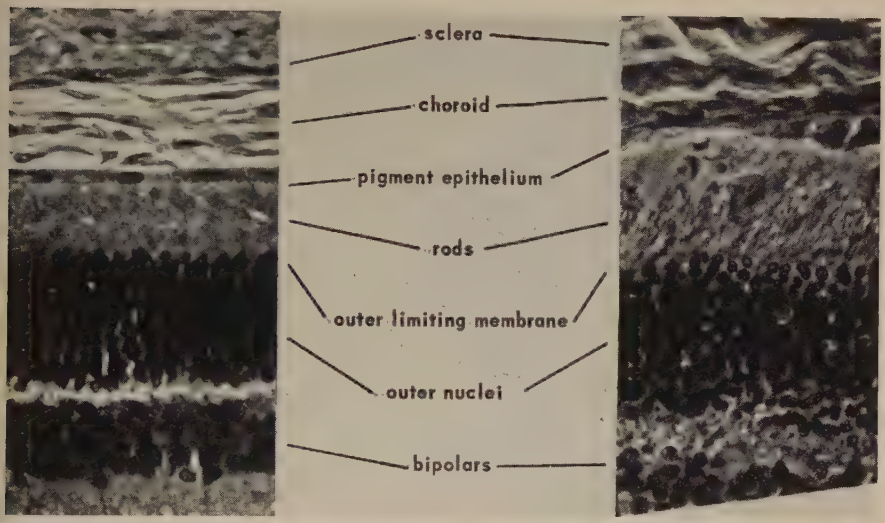

FIGURE 7. Effect of vitamin A deficiency on the structure of the rat retina. After 6 weeks on a vitamin A-deficient diet, the retina appears entirely normal. After 8 weeks it has deteriorated markedly: only traces of the pigment epithelium remain; the layer of rods, particularly the outer segments, is disintegrating; and the external limiting membrane has disappeared.

Neo-b vitamin A, although looked for, has never yet been found outside the eye tissues.

We have attempted to correlate to a degree the retinal histology with the biochemical and physiological changes described above. FIGURE 7 shows a section of the retina of an animal that had been on the deficient diet for six weeks. At this time the rhodopsin had declined to about 60 per cent of normal and the threshold had risen over 1.5 log units. The opsin content of the retina, however, was still normal, and the retinal histology appeared to be entirely normal.

At the right of FIGURE 7 is shown a retinal section in the eighth week of deficiency. By this time the opsin had fallen to about 60 per cent of normal. The retinal histology is seriously disturbed. The outer segments have lost their regular array—many seem to be missing, and others have a gnawed appearance. The external limiting membrane is gone, and only traces of the

pigment epithelium remain. With the loss of opsin, therefore, as we had anticipated, the outer segments of the rods deteriorate anatomically; but at this time other tissues in the retina also deteriorate, and the rat displays general signs of tissue decay elsewhere in the body.

This investigation has revealed an altogether orderly series of changes in vitamin A deficiency, and no evidence of the operation of any other factor in these changes. Following depletion of the stores of vitamin A in the liver, the level of vitamin A in the blood can no longer be maintained. As the level in the blood falls, the retinal content of rhodopsin declines; rhodopsin begins to lose its prosthetic group. This marks the onset of night blindness. For a time the retina retains all its opsin. Then the opsin content also begins to decline, and this marks the beginning of the anatomical deterioration of the retinal tissues.

We would suppose that the length of time required to cure night blindness is related to this program of changes. When the rhodopsin content has declined, but the opsin level is still normal, it is only necessary to admit vitamin A to the retina to have rhodopsin synthesized back to its normal level and the night blindness corrected. However, if the deficiency has progressed to the point of the loss of opsin and the associated deterioration of retinal histology, additional time is required to repair these more deep-seated changes, and the cure of night blindness must be correspondingly delayed.

What is the reason for the loss of opsin in late stages of vitamin A deficiency? Many experiments *in vitro* have taught us that opsin is a far less stable protein than rhodopsin. The attachment of the prosthetic group has a powerful stabilizing effect on this protein. It is possible that *in vivo* the loss of the prosthetic group makes opsin unstable, and that this is the primary reason for its loss in vitamin A deficiency.

It is important to recognize, however, that at the time when opsin is leaving the retina and the outer segments of the rods are deteriorating, other retinal tissues also are showing marked deterioration. The rat at this time loses weight and exhibits many signs of general tissue decay. Perhaps we have in these observations a suggestion of the general function of vitamin A in the organism. It may be that key structural proteins in many tissues are stabilized by combination with vitamin A or its derivatives; and that in vitamin A deficiency these proteins, like opsin, deteriorate, with the consequent histological deterioration of the tissues that contain them.

Acknowledgments

The authors are indebted to Toichiro Kuwabara and David G. Cogan, Howe Laboratory of Ophthalmology, Boston, Mass., for preparing the histological sections used in this investigation.

References

Bodansky, O., J. M. Lewis & C. Haig. 1941. The comparative value of the blood plasma vitamin concentration and the dark adaptation as a criterion of vitamin A deficiency. Science. **94**: 370.

Brenner, S. & M. C. H. Brooks. 1942. Relation of liver stores to the occurrence of early signs of vitamin A deficiency in the white rat. J. Nutrition. **23:** 459.

Hecht, S. & J. Mandelbaum. 1940. Dark adaptation and experimental human vitamin A deficiency. Am. J. Physiol. **130:** 651.

Hume, E. M. & H. A. Krebs. 1949. Vitamin A requirements of human adults. Med. Research Council Brit.. Spec. Rept. Ser. **No. 264.**

Johnson, M. L. 1943. Degeneration and repair of the rat retina in avitaminosis. Am. Arch. Ophthalmol. **29:** 793.

Lewis, J. M., O. Bodansky, K. G. Falk & G. J. McGuire. 1942. Vitamin A requirements in the rat. J. Nutrition. **23:** 351.

Moore, T. 1953. *In* Symposium on Nutrition. R. M. Herriott, Ed. : 31–33. Johns Hopkins Press. Baltimore, Md.

Tansley, K. 1933. Factors affecting the development and regeneration of visual purple in the mammalian retina. Proc. Roy. Soc. London. **B114:** 79.

Wald, G. 1955. The photoreceptor process in vision. Am. J. Ophthalmol. **40:** 18.

Wald, G., L. Brouha & R. E. Johnson. 1942. Experimental human vitamin A deficiency and the ability to perform muscular exercise. Am. J. Physiol. **137:** 551.

Wald, G., H. Jeghers & J. Arminio. 1938. An experiment in human dietary night-blindness. Am. J. Physiol. **123:** 732.

Wald, G. & D. Steven. 1939. An experiment in human vitamin A deficiency. Proc. Natl. Acad. Sci. **25:** 344.

THE MECHANISM OF BLEACHING RHODOPSIN*

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It has been known for many years that light can cause *cis-trans* isomerizations about double bonds. The interconversion of maleic and fumaric acids[1, 2] is probably the best known example of this transformation. More recently, Hubbard and Wald[3] have shown that light promotes the interconversion of the *cis* and *trans* isomers of retinene (vitamin A aldehyde). Irradiation of any isomer of retinene results in the identical steady-state mixture of stereoisomers. The structures of the various *cis* and *trans* isomers of retinene are shown diagrammatically in FIGURE 1.

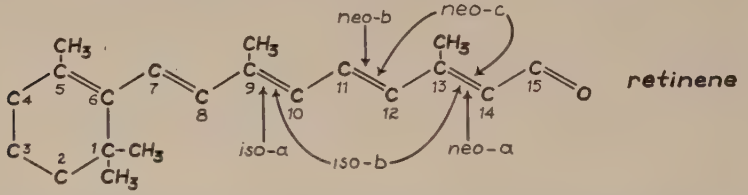

FIGURE 1. Structural formula of retinene showing the system of numbering carbon atoms. The structure as shown has the all-*trans* configuration. Arrows indicate the double bonds that are in *cis* configuration in the various *cis* isomers.

As is well known, rhodopsin is composed of a specific hindered *cis* isomer of retinene—neo-b (11-*cis*)[4]—joined to the colorless protein, rod opsin or scotopsin. Another *cis* isomer of retinene—iso-a (9-*cis*)—combines with rod opsin to form the artificial pigment, isorhodopsin.[3] None of the other isomers reacts with opsin.[3] Opsin, therefore, is stereospecific in its reactions with retinene.

Cattle rhodopsin absorbs maximally at 498 mμ; isorhodopsin at 487 mμ. The chromophores of the two pigments therefore differ, as they retain the *cis* configurations of their retinene precursors—11-*cis* in rhodopsin, 9-*cis* in isorhodopsin. If either pigment is bleached by thermal denaturation, the retinene is released in the appropriate *cis* configuration.[5] However, both pigments are bleached by light to a mixture of all-*trans* retinene and opsin. Bleaching by light, therefore, involves the stereoisomerization of the retinene chromophore to the all-*trans* configuration.

The interconversion of *cis* and *trans* isomers of retinene, while attached as chromophores to opsin, was first shown to occur with squid rhodopsin.[6] When squid rhodopsin at 5° C. and about *p*H 6 is exposed to light, it does not

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† Fellow in Cancer Research of the American Cancer Society, New York, N. Y.

bleach (that is, decolorize). Instead, the extinction of the solution rises and the absorption maximum shifts to somewhat longer wave lengths. These changes are associated with the conversion of squid rhodopsin (λ_{max} 493 mμ; ϵ_{max} 40,600) to another red compound, squid metarhodopsin, which has its absorption maximum at somewhat longer wave lengths (λ_{max} 500 mμ) and has a much higher molar extinction (ϵ_{max} 60,000) than squid rhodopsin.

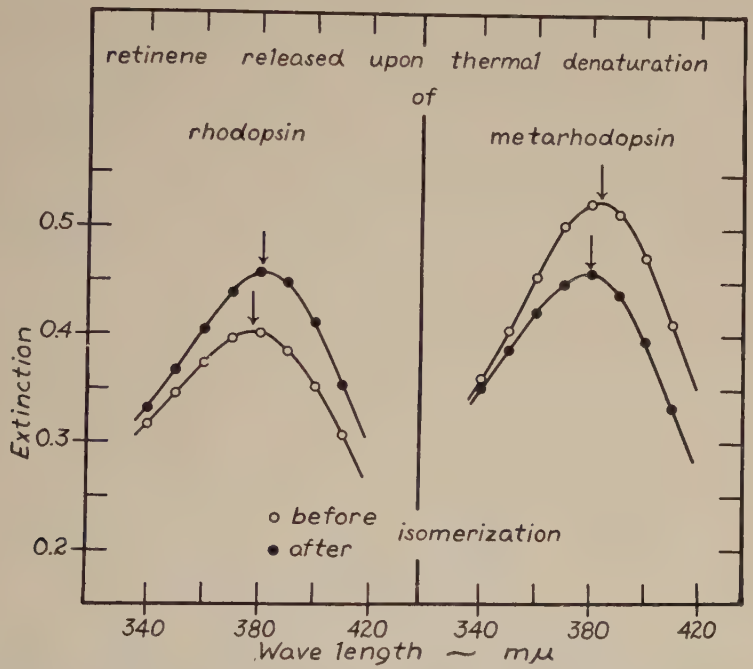


FIGURE 2. Stereoisomeric configurations of retinene released by thermal bleaching of squid rhodopsin (left) and metarhodopsin (right) in 2 per cent digitonin, *p*H 6.3. To minimize thermal isomerization of the products of bleaching,[5] rhodopsin and metarhodopsin are denatured by immersing them in a 70° C. water bath for 30 sec., and the products chilled immediately on ice. On irradiation with a white isomerizing light (160-watt tungsten filament lamp with Corning filters 3060 + 3966), the retinene liberated from rhodopsin rises in extinction and λ_{max} shifts to longer wave lengths (left), whereas the retinene liberated from metarhodopsin exhibits opposite changes (right).

Metarhodopsin is also photosensitive, and is converted by light back to rhodopsin. Starting with either squid rhodopsin or metarhodopsin, light therefore forms a mixture of both.

The chromophore of squid metarhodopsin has the all-*trans* configuration; that of rhodopsin is neo-b (11-*cis*). This can be demonstrated by irradiating the products of thermal bleaching of the two compounds with white light, which isomerizes retinene to a steady-state mixture of isomers.[3, 7] As shown in FIGURE 2, the retinene derived from rhodopsin rises in extinction, and λ_{max} shifts to longer wave lengths, indicating an isomerization pre-

dominantly from *cis* to *trans*, whereas the retinene derived from metarhodopsin exhibits the opposite behavior. As shown in FIGURE 3, the retinene released from rhodopsin can be identified further as the neo-b isomer by its capacity to form cattle rhodopsin (λ_{max} 498 mμ) when mixed with cattle opsin. The interconversion of squid rhodopsin and metarhodopsin by light,

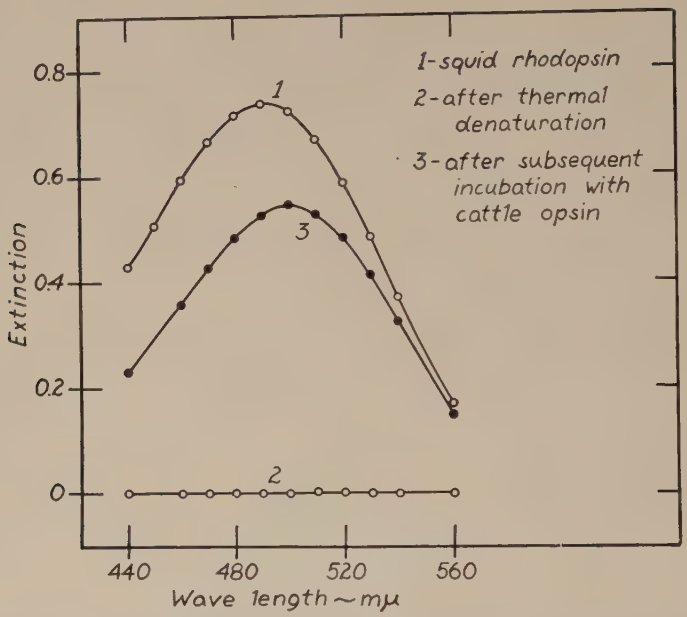


FIGURE 3. Stereoisomeric configuration of retinene released by thermal bleaching of squid rhodopsin. Squid rhodopsin in 2 per cent digitonin (spectrum 1; λ_{max} 493 mμ) was denatured by immersing it 30 sec. in a 70° C. water bath, and the product chilled on ice. This treatment bleached the rhodopsin, as shown in spectrum 2. Upon incubating the product with excess cattle opsin in the dark for 3 hours, it formed cattle rhodopsin (spectrum 3; λ_{max} 498 mμ). Spectrum 1 is a direct spectrum. Spectra 2 and 3 are difference spectra measured in the presence of excess hydroxylamine. However, such difference spectra are equivalent to absorption spectra at wave lengths longer than about 440 mμ. Due to partial thermal isomerization of the neo-b retinene released during thermal bleaching,[5] only 74 per cent as much rhodopsin is regenerated (spectrum 3) as was present initially (spectrum 1).

therefore, involves the stereoisomerization of their retinene chromophores while still attached to opsin.

Squid metarhodopsin is a *p*H indicator, red (λ_{max} 500 mμ) in neutral and mildly acid solution, and yellow (λ_{max} 380 mμ) in alkaline solution. The interconversion of the two forms proceeds in the dark, and involves only the reversible dissociation of one hydrogen ion from an acid-binding group with *p*K 7.7.[6] The two forms, called *acid* and *alkaline metarhodopsin*, both contain all-*trans* retinene. The absorption spectra of squid rhodopsin and of the two forms of metarhodopsin are shown in FIGURE 4.

The isomerization of acid metarhodopsin by light is stereospecific and yields only rhodopsin. The isomerization of alkaline metarhodopsin is less specific, yielding a mixture of rhodopsin and isorhodopsin.[6]

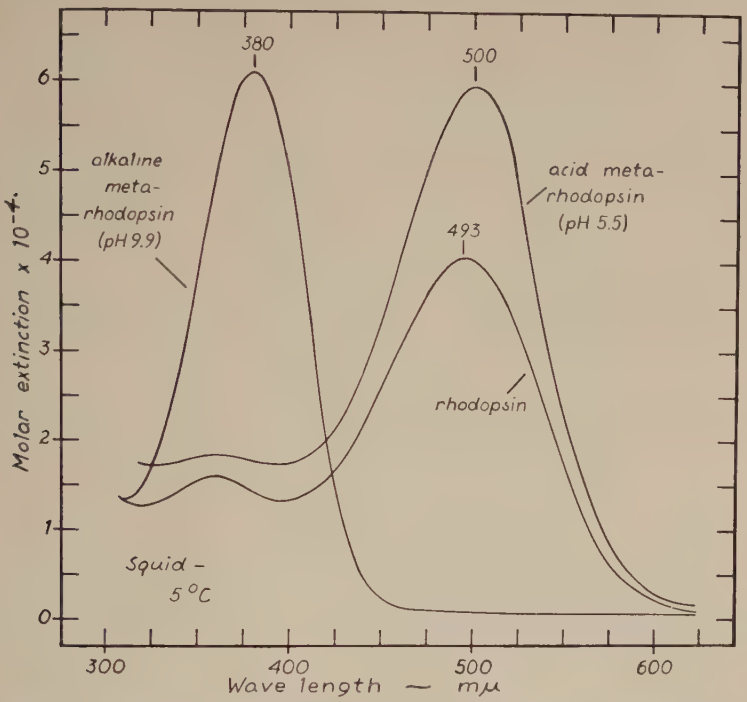


FIGURE 4. Absorption spectra of squid rhodopsin; alkaline metarhodopsin produced by irradiating rhodopsin with orange light at *p*H 9.9; and acid metarhodopsin produced by acidification of alkaline metarhodopsin to *p*H 5.5 in the dark. Solvent: 2 per cent aqueous digitonin.

The reactions described thus far can be summarized as follows:

$$\text{rhodopsin} \overset{\text{light}}{\rightleftharpoons} \text{acid metarhodopsin} \overset{-H^+}{\underset{+H^+}{\rightleftharpoons}} \text{alkaline metarhodopsin} \begin{cases} \xrightarrow{\text{light}} \text{rhodopsin} \\ \xrightarrow{\text{light}} \text{isorhodopsin} \end{cases} \quad (1)$$

Squid metarhodopsin is stable up to about 20° C. Above this temperature, it slowly hydrolyzes to retinene and opsin. For this reason it is unlikely that appreciable retinene is produced in the living squid eye.[6] Vision must be triggered by the photoisomerization of rhodopsin to metarhodopsin.

Is this simple description of the photochemistry of squid rhodopsin applicable also to vertebrate rhodopsins? It has been known for a number of years[8, 9] that below −15° C. cattle rhodopsin does not bleach to retinene and

opsin; instead, it is converted to orange intermediates, which Wald *et al.*[9] called lumi- and metarhodopsin. When these intermediates are warmed above −15° C., one obtains mixtures containing roughly one part of retinene and opsin, and one part of rhodopsin and isorhodopsin.[9, 10] It was assumed earlier that all these products are formed from the orange intermediates by

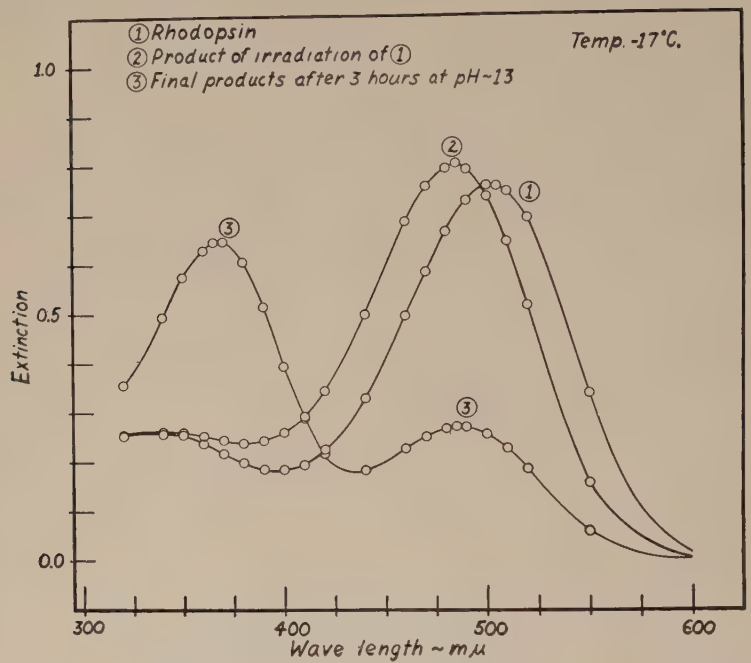


FIGURE 5. Action of light on cattle rhodopsin. Cattle rhodopsin at −17° C. (spectrum 1) is irradiated with white light (tungsten filament lamp, with Jena BG 18 heat filter) until the spectrum stops changing. The product of irradiation (spectrum 2) is not a homogeneous compound, as is shown by raising the *p*H in the dark to about 13 with 9 N sodium hydroxide. This decomposes part of the mixture. After 3 hours, the solution contains about 2 parts retinene and opsin, and one part rhodopsin and isorhodopsin (spectrum 3). Solvent: 0.5 per cent digitonin in a 1:1 glycerol-water mixture.

thermal reactions in the dark. The experiments to be described, however, necessitate a reinterpretation of these observations.

Cattle rhodopsin, like squid rhodopsin, is isomerized by light to all-*trans* metarhodopsin, which in turn is photoisomerized to a steady-state mixture of isomers, including rhodopsin and isorhodopsin. Consequently, irradiation of rhodopsin below −15° C. produces mixtures of metarhodopsin (or lumirhodopsin*), rhodopsin, and isorhodopsin. Upon warming, metarhodopsin

* These experiments have dealt primarily with metarhodopsin, although light in fact converts rhodopsin to lumirhodopsin. Lumirhodopsin is stable below about −45° C. and seems to make a fleeting appearance when rhodopsin or isorhodopsin is irradiated at −20° C. However, the differences between lumi- and metarhodopsin are not yet understood, and are at present under investigation.

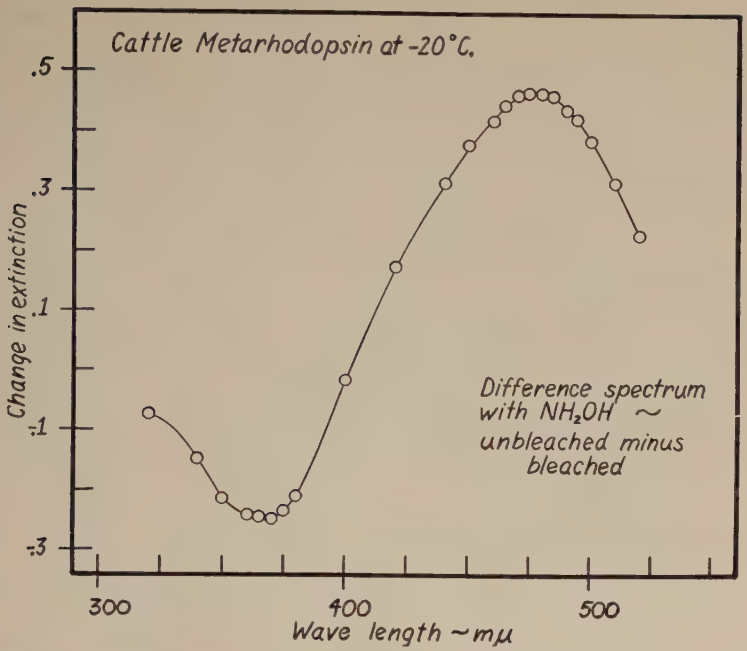
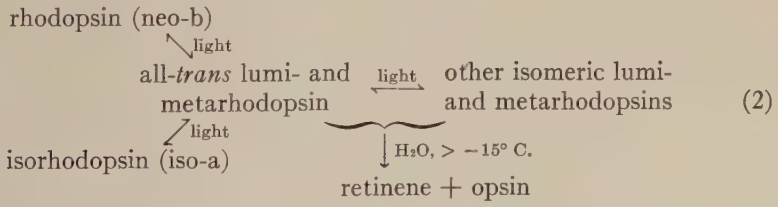


FIGURE 6. Difference spectrum of cattle metarhodopsin at −20° C. Solvent: 0.3 per cent digitonin in a 2:1 glycerol-water mixture. Rhodopsin at −20° C. is irradiated exhaustively with monochromatic light at 500 mμ. Hydroxylamine is then added to a final concentration of about 0.1 M and the spectrum measured. The solution is now warmed to room temperature in the dark. This bleaches the metarhodopsin, converting its chromophore to retinene oxime. After about three quarters of an hour, the solution is recooled to −20° C. and the spectrum measured again. The difference between the two spectra is shown.

is hydrolyzed to predominantly all-*trans* retinene and opsin, while the rhodopsin and isorhodopsin components of the mixture remain. These reactions can be summarized as follows:*



(2)

This scheme rests on the experiments summarized in FIGURES 5 and 8. Rhodopsin at −17° C. (FIGURE 5, curve 1) is irradiated with white light until there is no further change in spectrum. The product (FIGURE 5, curve 2) has a somewhat higher extinction than rhodopsin, and its λ_{max} is shifted to shorter

* We have recently learned that W. A. Hagins has proposed a similar scheme on the basis of entirely different experiments.[11]

wave lengths. Curve 2 is, in fact, the spectrum of the photo-steady-state mixture of metarhodopsin, rhodopsin, and isorhodopsin. This is most readily shown in the following way. While keeping the solution cold (−17° C.), sodium hydroxide is added until the *p*H rises to about 13. Under

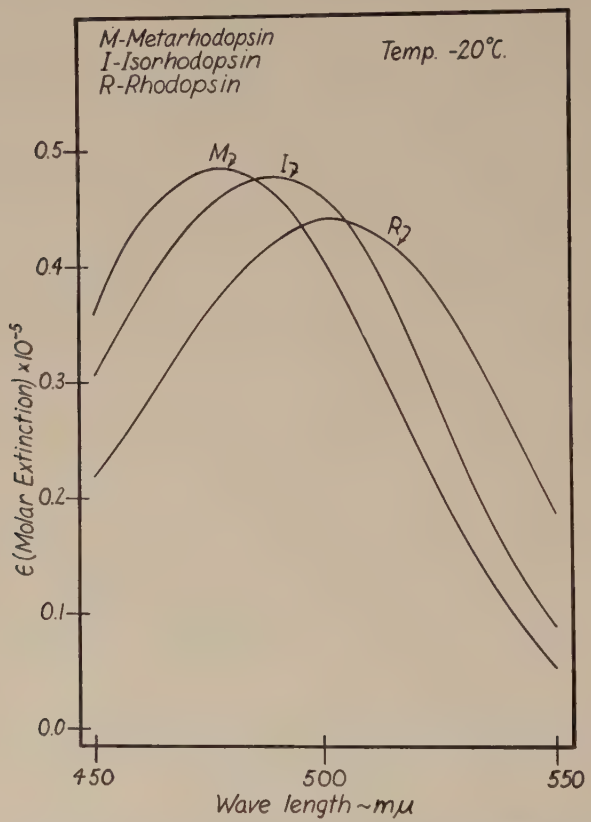


FIGURE 7. Spectra of cattle rhodopsin, metarhodopsin, and isorhodopsin at −20° C. Solvent: 0.3 per cent digitonin in a 2:1 glycerol-water mixture. All three are difference spectra measured in the presence of excess hydroxylamine, which combines with retinene to form retinene oxime. Since retinene oxime has essentially no absorption at wave lengths longer than about 440 mμ, the difference spectra above this wave length are identical with the absorption spectra. The molar extinctions of rhodopsin and isorhodopsin are calculated from the molar extinctions in this solvent system at room temperature (ϵ_{max} 42,000 and 43,000) by cooling solutions of known extinction to −20° C. and remeasuring their absorption spectra. The data for metarhodopsin are averages from the three experiments shown in FIGURE 8, and obtained by subtracting spectrum 3 of FIGURE 8 from spectrum 2.

these conditions, metarhodopsin hydrolyzes almost completely in about 3 hours, leaving a mixture of rhodopsin and isorhodopsin, and retinene and opsin (FIGURE 5, curve 3). The decay of extinction at 485 mμ is approximated by the superposition of the unimolecular decay curves of rhodopsin

and isorhodopsin measured at the same *p*H and temperature, with that of a third, far more labile component. Under the present experimental conditions, the latter has a half life of about 15 minutes, while rhodopsin and isorhodopsin decay with half lives of several hours. This labile component is metarhodopsin. Its difference spectrum at $-20°$ C. is shown in FIGURE 6.*

As one would expect on the basis of the formulation presented in EQUATION 2, the experiment shown in FIGURE 5 yields the same mixtures (curves 2 and 3), whether one starts with rhodopsin or with isorhodopsin.

The spectra of rhodopsin, isorhodopsin, and metarhodopsin are similar in shape. However, their absorption maxima (λ_{max}) and molar extinctions differ, as shown in FIGURE 7. The relative probabilities with which the three compounds capture quanta of a given wave length are, of course, governed by their molar extinctions at that wave length. The steady-state mixtures therefore vary somewhat in composition, depending on the wave length of light used to irradiate them. Thus green light, absorbed preferentially by rhodopsin, favors the formation of metarhodopsin, whereas blue light, absorbed preferentially by metarhodopsin, produces a steady-state mixture containing more rhodopsin.

This qualitative argument can be formulated more rigorously. For the coupled reactions,

$$\text{rhodopsin} \overset{\text{light}}{\longleftrightarrow} \text{metarhodopsin} \overset{\text{light}}{\longleftrightarrow} \text{isorhodopsin} \tag{3}$$

we can write the following expressions for the concentrations of rhodopsin, R, metarhodopsin, M, and isorhodopsin, I, at the photosteady state:

$$\frac{(M)}{(R)} = \frac{\epsilon_r \gamma_{r\to m}}{\epsilon_m \gamma_{m\to r}} \tag{4}$$

and

$$\frac{(M)}{(I)} = \frac{\epsilon_i \gamma_{i\to m}}{\epsilon_m \gamma_{m\to i}} \tag{5}$$

in which ϵ_r, ϵ_m, and ϵ_i are the molar extinctions of rhodopsin, metarhodopsin, and isorhodopsin at the wave length of irradiation, and $\gamma_{r\to m}$, $\gamma_{m\to r}$, $\gamma_{i\to m}$, and $\gamma_{m\to i}$, the quantum efficiencies for their interconversions in the directions indicated by the subscripts. The derivation of these equations is described in more detail elsewhere.[12]

The results of several experiments in which the photosteady state was produced by irradiating rhodopsin at 450, 500, or 550 mμ are shown in FIGURE 8. The steady-state concentrations of rhodopsin, metarhodopsin, and isorhodopsin are given in TABLE 1. From these data, we have calculated

* It must be noted that we are here redefining the terms lumi- and metarhodopsin. These terms, as previously used,[9] refer to the product of irradiation (FIGURE 5, curve 2), hence, not to single compounds, but to steady-state mixtures of chromoproteins, including photoregenerated rhodopsin and isorhodopsin. However, we are now (EQUATION 2) defining lumi- and metarhodopsin as the *labile* fraction of the mixture of stereoisomeric chromoproteins, the fraction that hydrolyzes to retinene and opsin in the dark above about $-15°$ C.

the relative quantum efficiencies for the interconversions of metarhodopsin with rhodopsin and with isorhodopsin.

Expressing all quantum efficiencies relative to the quantum efficiency for converting rhodopsin to metarhodopsin ($\gamma_{r\to m}$), arbitrarily taken as 1, we can

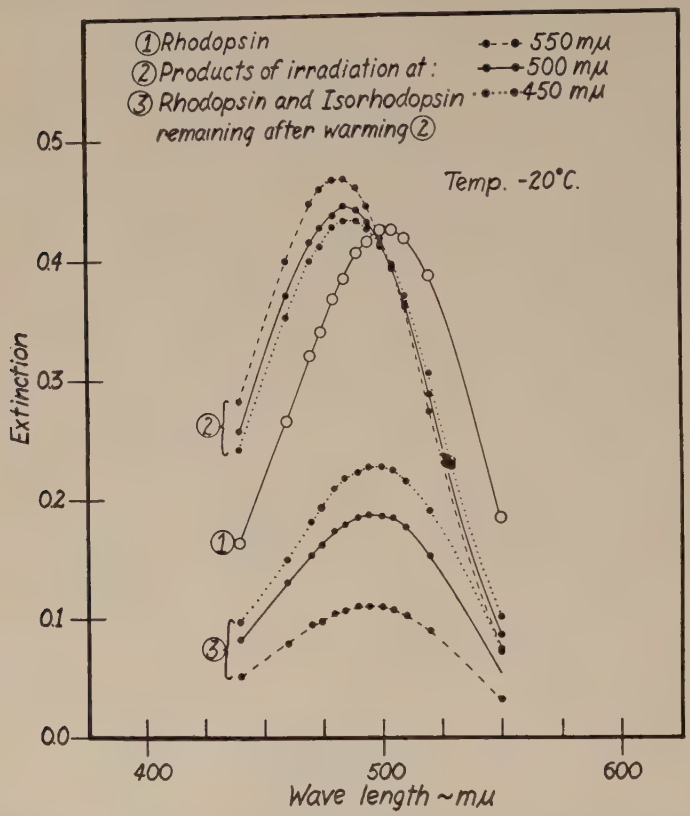


FIGURE 8. Steady-state mixtures of metarhodopsin, rhodopsin, and isorhodopsin produced by irradiating cattle rhodopsin at −20° C. with monochromatic lights of 450, 500, and 550 mμ (half-width = 11 mμ). Rhodopsin (spectrum 1) is irradiated until there is no further change in spectrum (spectra 2). Hydroxylamine is now added to a final concentration of about 0.1 M, and the solutions are warmed to room temperature in the dark to convert metarhodopsin to retinene oxime. The solutions are then recooled to −20° C., the spectra measured and corrected for dilution by hydroxylamine (spectra 3). The three conditions of irradiation yield different mixtures (spectra 2) containing different amounts of rhodopsin and isorhodopsin (spectra 3). The compositions of the mixtures are summarized in TABLE 1. Solvents as in FIGURES 6 and 7.

calculate $\gamma_{m\to r}$ from EQUATION 4. In order to calculate $\gamma_{m\to i}$, however, we need to know $\gamma_{i\to m}$. This is determined as follows. Rhodopsin and isorhodopsin are bleached with dim white light under conditions in which the light reaction is rate limiting. The rate of bleaching of each compound is then governed only by the rate at which its chromophore is isomerized to the

TABLE 1

COMPOSITIONS OF STEADY-STATE MIXTURES PRODUCED BY IRRADIATING RHODOPSIN AT −20° C. WITH MONOCHROMATIC LIGHTS OF VARIOUS WAVE LENGTHS*

Compound	Steady-state concentrations following irradiation at		
	450 mμ	500 mμ	550 mμ
Rhodopsin	34	25	11
Isorhodopsin	17	17	14
Metarhodopsin	49	58	75

* Half-width = 11 mμ. Concentrations, given in percentages, are calculated from the data shown in FIGURE 8: rhodopsin and isorhodopsin from spectra 3, by a method similar in principle to one previously described;[13] metarhodopsin from the differences between the initial concentration of rhodopsin (spectrum 1) and the final concentrations of rhodopsin and isorhodopsin (spectra 3).

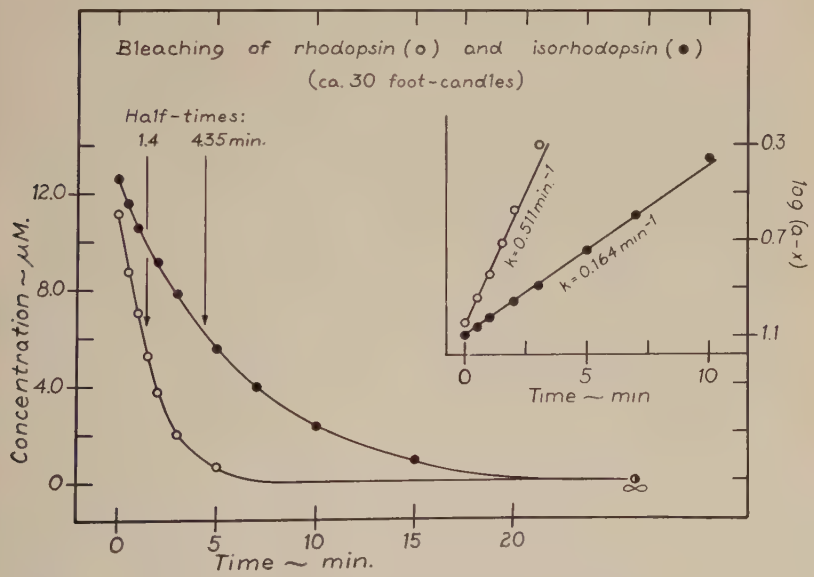


FIGURE 9. Relative rates of photochemical bleaching of cattle rhodopsin and isorhodopsin at 25° C. Solvent: 1.6 per cent aqueous digitonin, 0.2 M in hydroxylamine. *p*H 6.3. The concentrations of rhodopsin and isorhodopsin are plotted as functions of the time of irradiation with dim white light—about 30 foot-candles. In the insert, the same data are plotted in terms of the equation for a monomolecular reaction. Although photochemical bleaching involves light and thermal, or "dark," reactions, the conditions of this experiment are such that the rate of bleaching is limited by the light reaction. The rate constants, which are labeled *k*, show that, under these conditions, rhodopsin bleaches about 3 times as fast as isorhodopsin.

all-*trans* configuration, as shown in EQUATION 2. Rhodopsin and isorhodopsin absorb white light to about the same extent. Under these conditions, their relative rates of bleaching therefore reflect the relative quantum efficiencies for their conversion to all-*trans* metarhodopsin. As shown in FIGURE 9, we find that the conversion of isorhodopsin to metarhodopsin has a relative quantum efficiency ($\gamma_{i\rightarrow m}$) of 0.3. With this value for $\gamma_{i\rightarrow m}$, we can calculate $\gamma_{m\rightarrow i}$ from EQUATION 5.

TABLE 2

RELATIVE QUANTUM EFFICIENCIES (γ) FOR CONVERTING CATTLE METARHODOPSIN TO RHODOPSIN AND ISORHODOPSIN BY IRRADIATION AT 450, 500, AND 550 mμ*

Wave length of irradiation (mμ)	Relative quantum efficiencies for the reactions	
	Metarhodopsin to rhodopsin	Metarhodopsin to isorhodopsin
450	0.4	0.09
500	0.5	0.1
550	0.5	0.09
Averages.....	0.5	0.09

* Calculated from the data presented in FIGURE 8 and TABLE 1, by means of EQUATIONS 4 and 5. The values obtained at the three wave lengths are averaged in the last line. However, it remains to be demonstrated whether these quantum efficiencies are independent of wave length and, therefore, whether it is legitimate to average them.

The results of these calculations are summarized in TABLE 2. They show that rhodopsin is the most photosensitive of the three pigments. This may be related to the fact that neo-b retinene in solution also is isomerized more readily than the other isomers.[14] It is tempting to speculate that this is the reason why this thermodynamically improbable, hindered *cis* isomer has been selected as the chromophore of all known visual pigments. TABLE 2 further shows that, once formed, metarhodopsin has about five times the probability of being isomerized to rhodopsin than to isorhodopsin. This is probably one reason for the failure of isorhodopsin to accumulate in the living eye.

To recapitulate: light acts upon rhodopsin, by isomerizing its chromophore, to form all-*trans* lumi- or metarhodopsin. Light can then go on to isomerize the chromophore of either compound to a steady-state mixture of geometrical isomers, all still attached to opsin. The neo-b component of this mixture is itself rhodopsin; the iso-a component is itself isorhodopsin. No thermal back reaction is needed to make these pigments, for they are regenerated by isomerization *in the light*. The remaining isomers—all-*trans*, and possibly others—constitute an isomeric set of lumi- or metarhodopsins, all of which are unstable when warmed in the presence of water, and hydrolyze to mixtures of retinene and opsin.

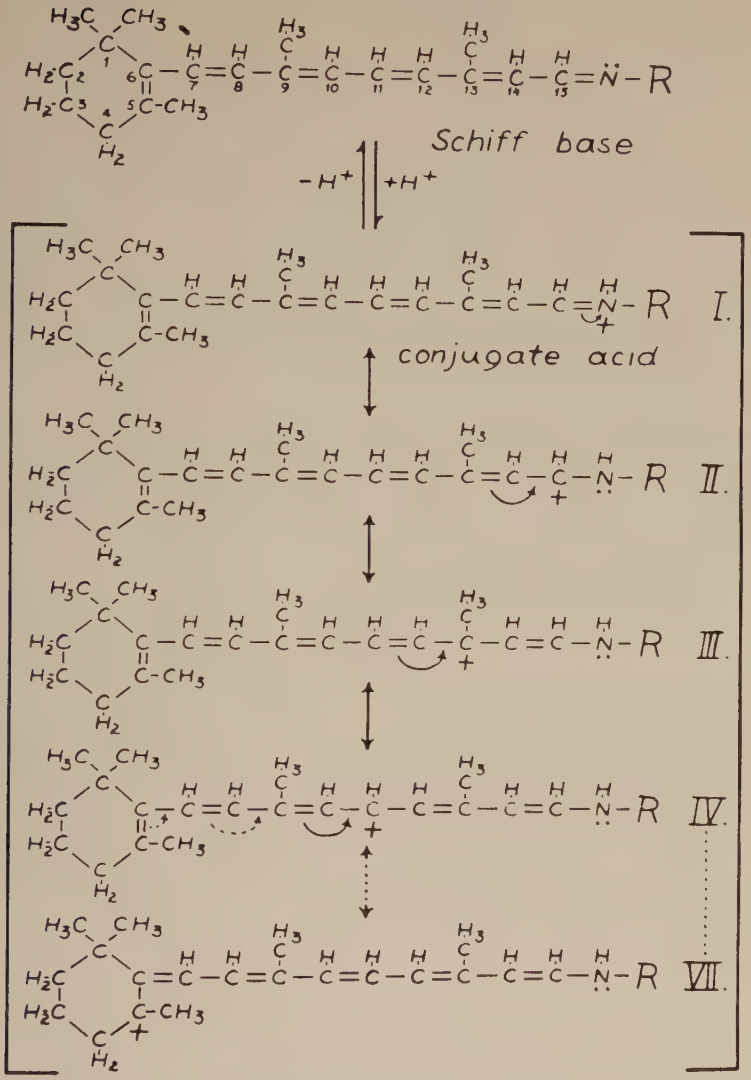


FIGURE 10. Schiff base of retinene, and the most stable limiting states of its conjugate acid. The conjugate acid is not represented accurately by any one of these structures, but is a resonance hybrid of structures I through VII.

Retinene is yellow (λ_{max} about 385 mμ). All the chromoproteins we have been discussing are orange or red (λ_{max} 480–500 mμ). What is the mechanism of the shift in spectrum that accompanies the combination of retinene with opsin?

Retinene is attached to opsin probably through a Schiff-base linkage, $R \cdot \underset{H}{C}{=}N \cdot R'$,[15, 16] formed by the condensation of its carbonyl group ($-\underset{H}{C}{=}O$)

with a specific amino ($—NH_2$) group on opsin. Experiments with model compounds[16-18] suggest that the chromophore is derived from the conjugate acid of this Schiff base, $R \cdot C(H)=\overset{+}{N}H \cdot R'$. Conjugate acids of model Schiff bases of retinene with aliphatic amino compounds, however, have absorption maxima at 440 to 450 mμ. In order to shift the absorption maximum toward a longer wave length, the opsin must in some way interact with the system of conjugated double bonds of the chromophore.[19]

Resonance theory predicts that the first excited state of the chromophore can be represented largely as a resonance hybrid of structures I through VII shown in FIGURE 10, whereas the ground state is almost exclusively structure I.* Therefore, the excited state of the chromophore has positive charge distributed over the entire conjugated system. One way in which opsin can interact with the chromophore and lower the energy of the first excited state is by stabilizing the positive charge through its own negatively charged groups, such as COO^-, O^-, or S^-. By stabilizing the first excited state, opsin can lower the energy of the transition from the ground state to the first excited state of the chromophore, and thus shift its absorption spectrum toward longer wave lengths (that is, smaller quanta).

The color of these chromoproteins is therefore related to the degree of interaction of the retinene chromophore with opsin. The interaction is strongest with neo-b (11-*cis*) retinene, and somewhat weaker with the iso-a (9-*cis*) isomer. Isomerization of either *cis* isomer to the all-*trans* configuration lowers the interaction, shifting the absorption spectrum toward shorter wave lengths, as in vertebrate metarhodopsins. The relative ease of hydrolysis of vertebrate metarhodopsins, compared with the *cis* chromoproteins—rhodopsin and isorhodopsin—is further evidence that the all-*trans* chromophore is bound to opsin less strongly than is the 9- or 11-*cis* chromophore. This interpretation is consistent with the observation that squid metarhodopsin, which has its λ_{max} at longer wave lengths than the vertebrate metarhodopsins, is also less readily hydrolyzed.

The mechanism of bleaching vertebrate rhodopsins is summarized schematically in FIGURE 11. We postulate that in rhodopsin the neo-b chromophore fits the chromophoric site on opsin. Light destroys this fit by isomerizing the chromophore to the all-*trans* configuration. This is presumably the reaction responsible for visual excitation. The orange all-*trans* chromoprotein, metarhodopsin, is readily hydrolyzed, and bleaches to retinene and opsin.

Neo-b retinene combines with rod opsin to form rhodopsin, with λ_{max} around 500 mμ. With cone opsin, it forms iodopsin with λ_{max} near 560 mμ. Furthermore, rhodopsins from different species differ in the precise position of their λ_{max}.[21, 22] In all these cases, the chromophore is derived from neo-b retinene. The spectroscopic differences must be due to differences in the opsins, which determine the degree of interaction with the chromophore, and thereby its color.

* A complete discussion of the theory of resonance as applied to problems of molecular spectra can be found in chapter 6 of *Resonance in Organic Chemistry*.[20]

To recaptitulate, rhodopsin is composed of the colorless protein, opsin, combined with a hindered *cis* isomer of the yellow carotenoid-derivative, retinene. We believe that the red color of rhodopsin arises in the following way. Retinene, through its carbonyl group, forms a Schiff base with a specific amino group on opsin. The chromophore is derived from the conjugate acid of this Schiff base. Negatively charged groups elsewhere on opsin stabilize the resonance forms of the chromophore that constitute its first excited state. This lowers the energy of the transition between the ground and first excited states, and shifts the color of the chromophore from yellow to red. The differences in color between rhodopsin, isorhodopsin, and

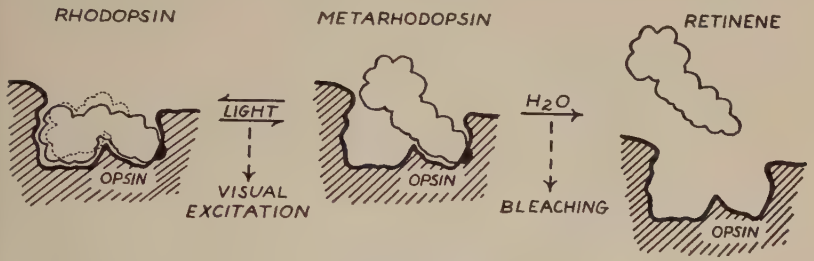


FIGURE 11. Hypothetical scheme showing the effect of light on vertebrate rhodopsins. In rhodopsin, the neo-b (11-*cis*) chromophore fits into the chromophoric site on opsin. This site is fitted also, although less well, by the iso-a (9-*cis*) chromophore of isorhodopsin, which is shown by the dotted line. Light isomerizes the *cis* chromophore to the all-*trans* configuration, thus decreasing the interaction between the chromophore and opsin. This is all-*trans* metarhodopsin, which hydrolyzes readily to retinene and opsin. The isomerization of rhodopsin to metarhodopsin is probably responsible for visual excitation, but bleaching is due to the hydrolysis of metarhodopsin.

metarhodopsin are due to differences in the fit between the three isomeric chromophores and the opsin surface. Similarly, differences in color between rhodopsins from various species, and between rhodopsin and iodopsin, are due to differences in interaction between the neo-b chromophore and the different opsins.

Acknowledgments

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References

1. WARBURG, E. 1919. Über den Energieumsatz bei photochemischen Vorgängen. IX. Photochemische Umwandlung isomerer Körper ineinander. Sitzber. preuss. Akad. Wiss. Physik. math. Kl. **33**: 960.
2. VAIDYA, B. K. 1930. Geometrical inversion by light. Proc. Roy. Soc. London. **A129**: 299.
3. HUBBARD, R. & G. WALD. 1952–1953. *Cis-trans* isomers of vitamin A and retinene in the rhodopsin system. J. Gen. Physiol. **36**: 269.

4. Oroshnik, W., P. K. Brown, R. Hubbard & G. Wald. 1956. Hindered *cis* isomers of vitamin A and retinene: the structure of the neo-b isomer. Proc. Natl. Acad. Sci. **42:** 578.
5. Hubbard, R. 1958–1959. The thermal stability of rhodopsin and opsin. J. Gen. Physiol. In press.
6. Hubbard, R. & R. C. C. St. George. 1956–1957. The rhodopsin system of the squid. J. Gen. Physiol. **41:** 501.
7. Hubbard, R., R. I. Gregerman & G. Wald. 1952–1953. Geometrical isomers of retinene. J. Gen. Physiol. **36:** 415.
8. Broda, E. E. & C. F. Goodeve. 1941–1942. The behavior of visual purple at low temperatures. Proc. Roy. Soc. London. **B130:** 217.
9. Wald, G., J. Durell & R. C. C. St. George. 1950. The light reaction in the bleaching of rhodopsin. Science. **111:** 179.
10. Collins, F. D. & R. A. Morton. 1950. Studies in rhodopsin. III. Rhodopsin and transient orange. Biochem. J. **47:** 18.
11. Hagins, W. A. 1958. Ph.D. Thesis. Cambridge Univ. Cambridge, England.
12. Hubbard, R. & A. Kropf. 1958. The action of light on rhodopsin. Proc. Natl. Acad. Sci. **44:** 130.
13. Hubbard, R. 1955–1956. Retinene isomerase. J. Gen. Physiol. **39:** 935.
14. Hubbard, R. 1956. Geometrical isomerization of vitamin A, retinene and retinene oxime. J. Am. Chem. Soc. **78:** 4662.
15. Collins, F. D. 1953. Rhodopsin and indicator yellow. Nature. **171:** 469.
16. Pitt, G. A. J., F. D. Collins, R. A. Morton & P. Stok. 1955. Studies on rhodopsin. VIII. Retinylidenemethylamine, an indicator yellow analogue. Biochem. J. **59:** 122.
17. Morton, R. A. & G. A. J. Pitt. 1955. Studies on rhodopsin. IX. *p*H and the hydrolysis of indicator yellow. Biochem. J. **59:** 128.
18. Morton, R. A. & G. A. J. Pitt. 1957. Visual pigments. Fortschr. Chem. org. Naturstoffe. **14:** 245.
19. Hubbard, R. 1958. The chromophores of the visual pigments. Proc. Natl. Phys. Lab. U. K., Symposium No. 8 (Visual Problems of Colour). H. M. Stationery Office. In press.
20. Wheland, G. W. 1955. Resonance in Organic Chemistry. Wiley. New York, N. Y.
21. Wald, G., P. K. Brown & P. S. Brown. 1957. Visual pigments and depths of habitat of marine fishes. Nature. **180:** 969.
22. Wald, G. & P. K. Brown. 1958. Human rhodopsin. Science. **127:** 222.

EFFECT OF FLASH ILLUMINATION ON RHODOPSIN IN SOLUTION*

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Introduction

This paper describes some of the results obtained using the flash-photolysis technique[1] to study the photochemical behavior of rhodopsin in solution, a preliminary report of which appears elsewhere.[2] Subjecting a solution of rhodopsin to a short, intense flash of light permits nearly simultaneous excitation of the pigment molecules. Subsequent changes of the pigment molecules can then be studied by means of a fast spectrophotometric technique that permits the measurement of absorption changes only microseconds after illumination. In brief, the results we obtained using this method demonstrate that flash excitation of rhodopsin in solution produces the following: (1) an initial photoproduct having an absorption spectrum different from that of rhodopsin; (2) spontaneous and partial disappearance of this photoproduct (dark reactions) in several well-defined stages; and (3) a terminal and stable, but light-sensitive, photoproduct.

Since visual excitation must be initiated by photochemical events occurring immediately upon or shortly after photoexcitation, it seemed desirable to study the behavior of rhodopsin immediately following flash illumination.

Methods

Rhodopsin solutions used in these experiments were digitonin extracts of tanned rod outer segments prepared from dark-adapted cattle retinas.[3] Stock solutions were buffered at *p*H 7, and maintained at 0° C., and aliquots were introduced into a cylindrical absorption cell 5 cm. long and 1 cm. in diameter. Experiments were performed at room temperature (between 27 and 28° C.), as well as at lower and higher temperatures. Absorption spectra of all stock rhodopsin solutions were measured with a recording spectrophotometer.‡

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The filled absorption cell was placed in an optical system that permitted flash excitation of the pigment and the monitoring of changes in absorption during and after the flash excitation (FIGURE 1). The exciting flash was provided by four argon-filled flash tubes placed symmetrically within a reflector around the absorption cell and fired simultaneously (± 0.1 μsec.) by a trigger pulse. The total discharge energy was 64 joules. The duration of the flash, measured from initiation to half decay, was 2 μsec. At 6 μsec. the intensity was about 5 per cent of the peak. However, scattered light from a relatively weak, but prolonged, tail limited the resolution time to about 20 μsec. A collimated light beam from a zirconium arc, attenuated

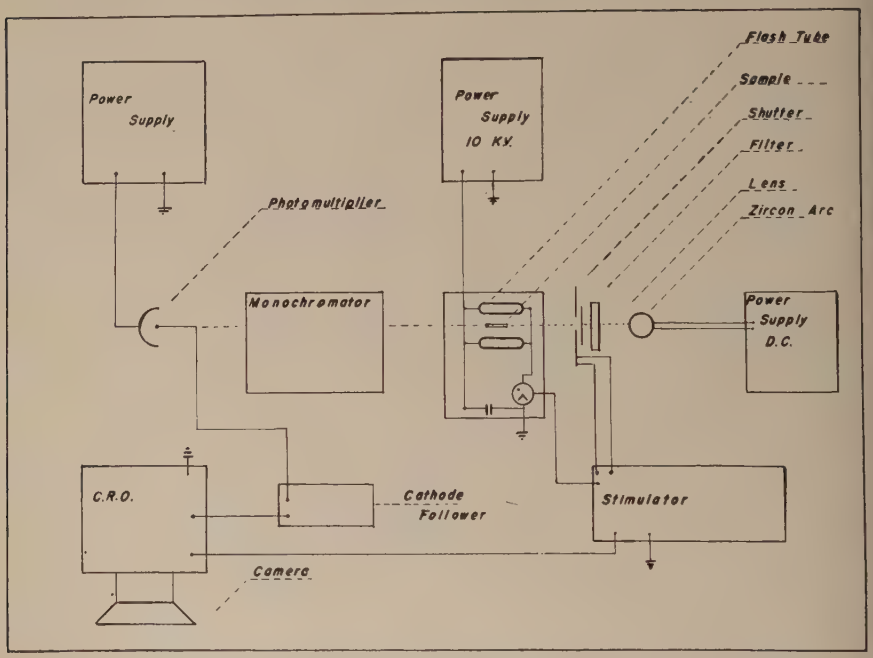

FIGURE 1. Schematic diagram illustrating the equipment and its arrangement.

by selected interference and neutral filters and controlled by a shutter, passed through the absorption cell, then through a monochromator and into a photomultiplier tube, the output of which produced vertical deflections of the cathode-ray trace. Tests with calibrated neutral filters established the fact that the oscillograph deflection was proportional to transmitted light intensity. The wave-length band passed by the monochromator varied from 2.0 mμ to 5.3 mμ. A switch activated by opening the shutter served to initiate two pulses, which were separated by an adjustable time interval; the first of these triggered the sweep of the cathode-ray trace, and the second initiated the exciting flash. A fresh sample of rhodopsin was used for each flash, of course. The bleaching of rhodopsin caused by the measuring beam was negligible.

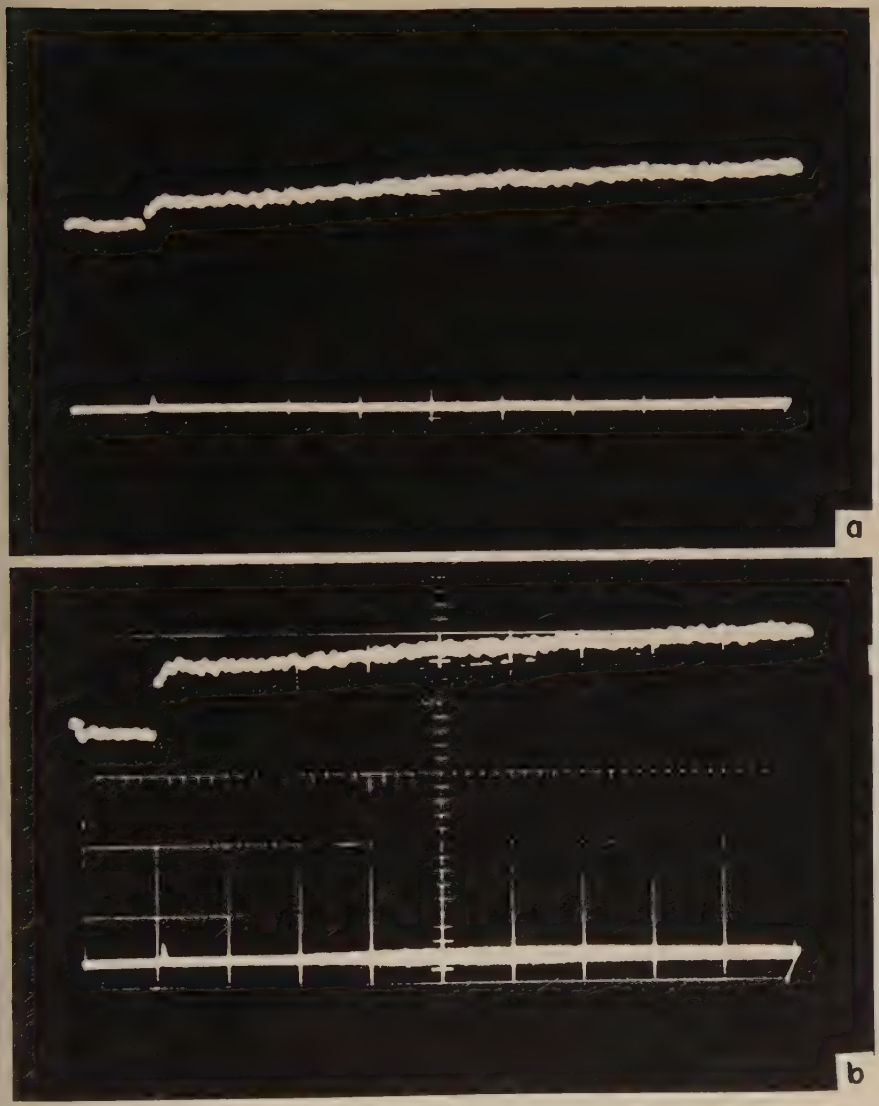

FIGURE 2. Oscillograms obtained (*a*) at 480 mμ, and (*b*) at 516 mμ, at a sweep speed of 4 msec. per cm. The vertical grid lines in *b* are 1 cm. apart. Traces are read from left to right. Both lower traces represent the base line (without analyzing light) for obtaining the level for zero-light transmission and for measuring the scattered light that reached the photomultiplier tube from the exciting flash (small spike at left of *b*). Both upper traces measure the light transmitted through the rhodopsin solution before and after flash excitation.

Results

The nature of the data obtained is indicated by the oscillograms in FIGURES 2 and 3. In FIGURE 2, for example, the upper trace in both frames measures the light transmitted through the absorption cell before and after the exciting flash, the occurrence of which is indicated by part of the discontinuity in the trace. The portion to the left of the discontinuity in each upper trace measures the light transmitted through the rhodopsin solution prior to

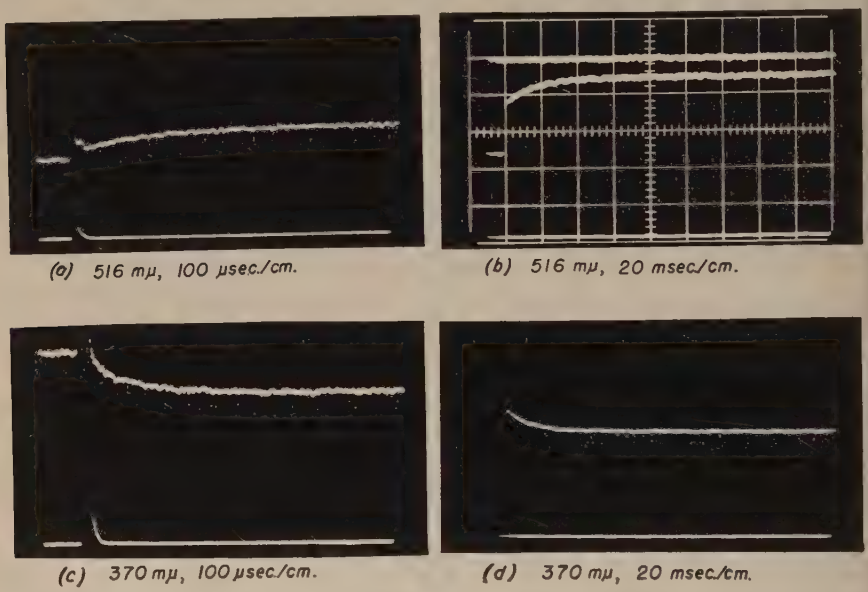

FIGURE 3. Oscillograms obtained at 516 mμ, (*a* and *b*), and at 370 mμ, (*b* and *c*), at two sweep speeds: 100 μsec. per cm. in *a* and *c*, and 20 msec. per cm. in *b* and *d*. The vertical grid lines in *b* are 1 cm. apart on the tube face; the third trace in *b* measures the light transmitted through the rhodopsin solution 1 minute after flash illumination. The lowest trace in each oscillogram is the zero-light transmission line on which is superimposed the scattered light from the flash, which is visible only in *a* and *c*. The other traces measure the light transmitted through the rhodopsin solution before and after flash illumination.

photoexcitation; the rest of the trace measures the light transmitted through the solution for about 36 msec. following excitation. The lower trace in both frames is obtained without the analyzing light to determine the base line (for zero light transmission) and to obtain a measure of the scattered light that reached the photomultiplier tube from the exciting flash alone.

The records in each oscillogram were projected onto graph paper, traced, and analyzed to yield the change in optical density of the photoexcited rhodopsin solution as a function of time. Since the distance between the zero-transmission line and the tracing with shutter open measures the light transmitted through the rhodopsin solution, it is possible to compute the ratio of light transmitted prior to photoexcitation to light transmitted at any time following excitation. The logarithm of this ratio is the change in

optical density ΔD of the photoexcited rhodopsin solution. Adding this change in density ΔD at a given time and wave length to the original density of the rhodopsin sample taken from the absorption spectrum as measured before flashing, then gives the absolute optical density of the flashed solution at that corresponding time and wave length. Thus, one may reconstruct complete absorption spectra of the solution at various times after photoexcitation. To determine the ΔD for the initial photoproduct (time = zero),

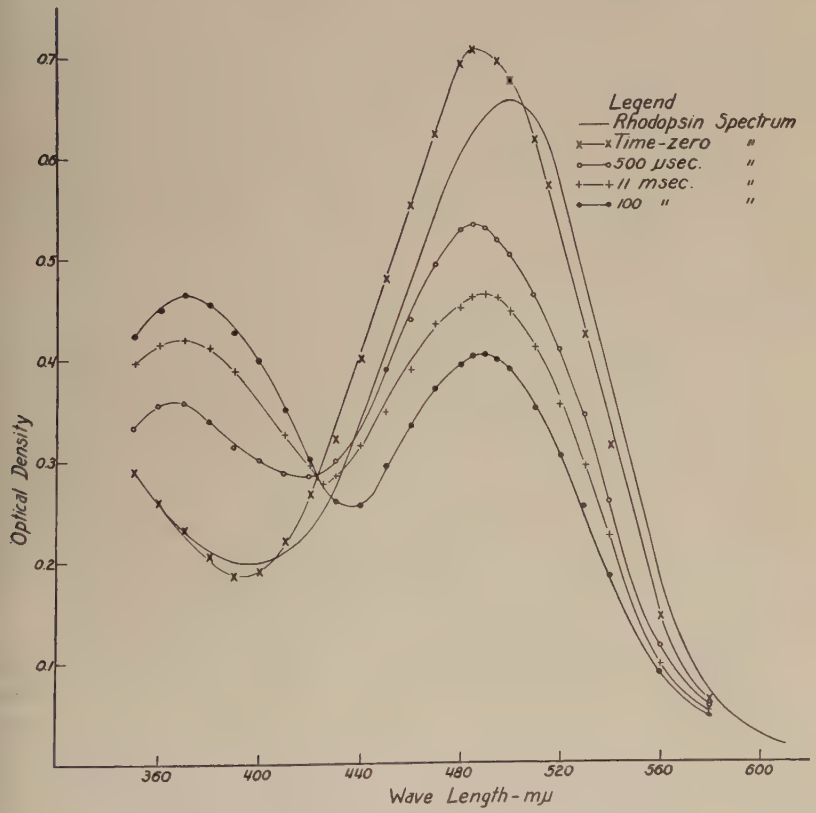

FIGURE 4. Absorption spectrum of rhodopsin prior to flash excitation—shown by the continuous line—and reconstructed absorption spectra of the pigment 0.5, 11 and 100 msec. after flash illumination. These times correspond approximately to the end of processes 1, 2, and 3, respectively.

the tracings for each wave length were extrapolated back to zero time (initiation of flash). This procedure corrects for the dark reaction during the 20 μsec. in which scattered light from the flash interferes with the measurement. The absorption spectra of photoexcited rhodopsin at various times after the flash are shown in FIGURES 4 and 5. It can be seen from inspection of FIGURES 2 and 3 that the spectral changes following flash excitation occur in several stages.

Immediately upon photoexcitation, the absorption spectrum of the pig-

ment changes; the initial photoproduct shows a shift in $\lambda_{max.}$ to 486 mμ, and has a higher peak absorption (see the x's in FIGURE 4) than the original rhodopsin. During the next 500 μsec. there is a rapid decrease in absorption at 486 mμ and a correspondingly fast rise in absorption at 384 mμ. This is the first dark reaction (Process 1), which is recorded in FIGURE 3*a* and *c*, and is visible in FIGURE 2*a* and *b*. During the next 100 msec., certainly one further decrease ensues in absorption at 486 mμ, and perhaps two, with concomitant increases in the ultraviolet absorption. These two stages (Process 2 and Process 3) are difficult to separate, but may be seen in FIGURE 2

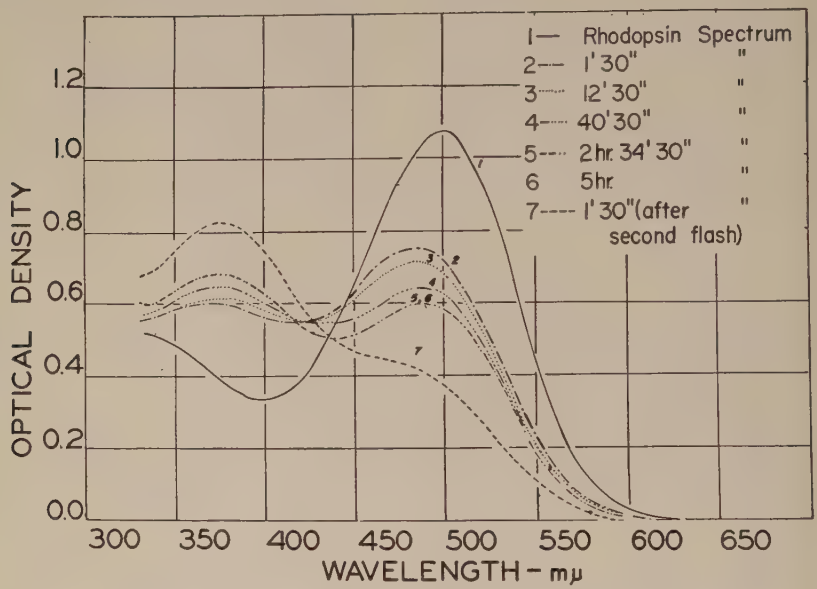

FIGURE 5. Absorption spectrum of rhodopsin prior to flash illumination and absorption spectra of the pigment after flash excitation. The spectra were obtained with a recording spectrophotometer at various times after photoexcitation. The times given indicate the beginning of the trace at 650 mμ. Curve 7 represents the absorption spectrum 1.5 minutes after the solution measured in curve 6 was exposed to another flash of light. The changes in curves 2 through 5 represent Process 4.

(λ = 516 mμ) with a suggestion of a change of slope between them. At somewhat slower sweep speeds, as in (FIGURE 3*b* and *d*) Process 1 and Process 3 are very well defined. The small effect that we call Process 2 is consistently present in records taken at different wave lengths, and we therefore include it as a distinct stage in the over-all reaction. A further very slow bleaching at 486 mμ then occurs (Process 4) over the next 2 hours, with another corresponding rise at about 384 mμ. The final dark-stable solution shows a $\lambda_{max.}$ at 490 mμ (difference spectrum $\lambda_{max.}$ = 492 mμ) and has about half the peak absorption of the original rhodopsin solution. Exposure of this stable mixture to a second flash produces a further reduction in absorption in the visible spectrum and an increase in absorption in the near ultraviolet.

Approximately the same sequence of slower changes takes place as those described above, including a shift of the absorption maximum of the flashed product toward longer wave lengths. The final product has $\lambda_{max.} = 490$ mμ and an extinction approximately half again that of the solution before the flash.

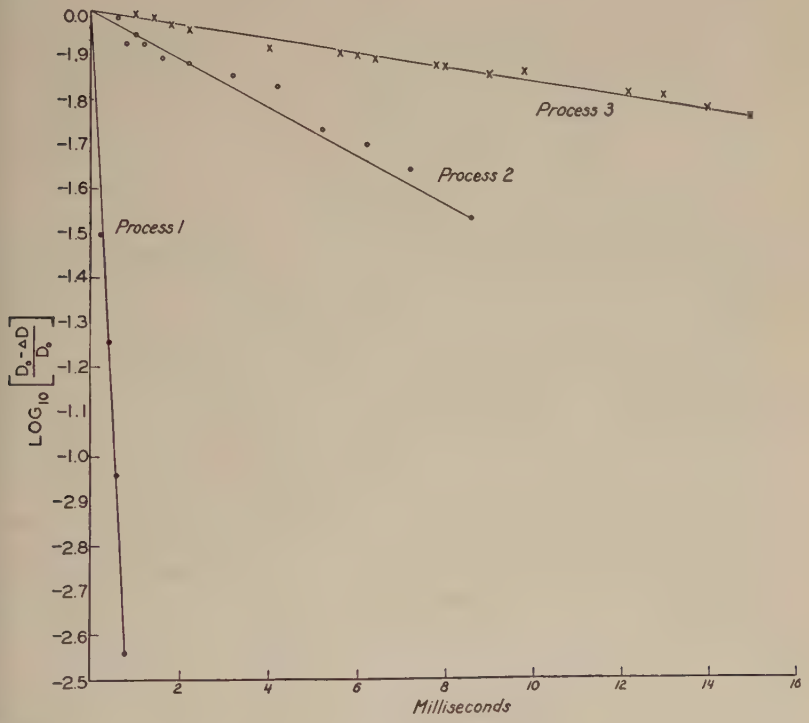

FIGURE 6. An analysis of the changes in light transmission through solutions of rhodopsin following flash excitation. The ordinate is $\log_{10}\left(\frac{D_0 - \Delta D}{D_0}\right)$, where D_0 is the total change in density for each process, and ΔD is the change in density at any time t during the process. The data for Processes 1, 2, and 3 were obtained from oscillograms employing sweep speeds of 100 μsec., 4 msec., and 20 msec. per cm., respectively. Zero time for Process 3 is actually 15 msec. after onset of the flash; for Process 2, 4 msec.; and Process 1 begins at zero time.

Within the precision of these data, Processes 1 and 3 appear to be first-order. We call ΔD the change in optical density at any time after the start of reaction and D_0 the total *change* in density for each process. These quantities are defined for *each* stage of the reaction, starting at a time when a preceding (much faster) reaction has been essentially completed, and ending before the next (much slower) reaction has progressed to any appreciable extent. By proper choice of sweep speeds and wave lengths, processes 1 and 3 may well be separated. However, as pointed out earlier, it is difficult to

resolve Process 2. The plots of log $\left(\frac{D_0 - \Delta D}{D_0}\right)$ are given in FIGURE 6. The linearity of these plots, in particular for Process 1 and Process 3, establish the first-order character of these reactions. The kinetics of Process 4 are more complex and require further analysis.

Experiments performed with rhodopsin at temperatures both above and below room temperature indicate that the rates of the decay processes are markedly dependent on temperature. The half life of Process 1 is 9.2×10^{-5} sec. at 28° C. and 1.4×10^{-5} sec. at 37.6° C.; the half life of Process 2 is 2.5×10^{-3} sec. at 28° C. and 1.1×10^{-4} sec. at 37.6° C.

Discussion

The initial photoproduct. The initial photoproduct produced by flash illumination of rhodopsin is a mixture consisting of one fraction that spontaneously decomposes in the dark (Fraction 1), and a second photosensitive fraction that is stable in the dark (Fraction 2). This result is in agreement with the recent findings of Hubbard and Kropf[4] and with earlier work,[5, 6] and it is probable that the dark-stable fraction is a mixture of rhodopsin and isorhodopsin. The fact that Fraction 1 decomposes at different rates indicates that it, too, is a mixture of pigment molecules, rather than a homogeneous population. In our experiments, then, the initial event following flash excitation of rhodopsin in solution is the formation of a photoproduct of considerable complexity.

The spectral absorption characteristics of the initial photoproduct resemble those of the intermediate photoproduct called metarhodopsin by Wald *et al.*,[6] and this is probably analogous to the transient orange of Lythgoe[7] and Broda and Goodeve.[8] Wald and his co-workers[6] and Hubbard and Kropf[4] have described another intermediate, called lumirhodopsin which, under certain conditions, precedes the appearance of metarhodopsin. In our experiments, we have obtained no evidence for the existence of lumirhodopsin, but it must be pointed out that our instruments were blinded to any absorption changes in the pigment solution during the 20 μsec. or less occupied by the exciting flash. We partially extended our look into this forbidden period by performing flash experiments at 4° C., but detected only the properties attributed to the initial photoproduct. It must be emphasized, however, that what we call the initial photoproduct has, by virtue of the analysis, the spectral characteristics of the product existing 20 μsec. after onset of the flash, extrapolated to zero time. Recently,[4] the terms lumirhodopsin and metarhodopsin have been redefined to include only that fraction of the initial photoproduct that is unstable in the dark. Since this dark-unstable fraction seems to be a mixture of pigment molecules having different properties, it may be wise to re-examine this terminology.

Significance of the decay processes. The results of our experiments demonstrate that the components of the dark-unstable fraction of the initial photoproduct conveniently decompose at different rates. Four discrete rates of decomposition have been identified (Processes 1, 2, 3, and 4) of which only Process 2 is subject to some question. These observations suggest that the

dark-unstable fraction of the initial photoproduct consists of three or possibly four unstable species. One species (Process 1) decomposes within 100 μsec. at room temperature; another (Process 2) decomposes more slowly, requiring 10 to 15 msec. for completion; a third (Process 3) requires 100 msec. for completion; and a fourth species dissociates over a much longer time, about an hour.

Hubbard and Kropf[4] have introduced the idea that the dark-unstable fraction of the photoproduct formed upon illumination of rhodopsin consists of a steady-state mixture of stereoisomeric chromophores still attached to opsin. This hypothesis provides a reasonable interpretation for the three or four decay processes identified. According to Hubbard,[9] six isomers of retinene have been identified; two of these, neo-b and iso-a, when bound to the visual protein are dark-stable and form the rhodopsin and isorhodopsin of the initial photoproduct. The other isomers, therefore, may form the unstable chromophore-protein complexes in the initial photoproduct and dissociate at different rates. Attractive though this hypothesis is, direct experimental proof of the theory must still be advanced. At this point, any specific correlation between the separate decay processes observed here and the isomeric retinene species formed is impossible because we lack definite information about the likelihood of occurrence of the di-*cis* isomers of retinene under such conditions.

Flash photolysis of excised eyes. Although the data published by Hagins,[10] which were obtained from the flash photolysis of intact but excised rabbit eyes, permit only a restricted analysis, they indicate that our results are in essential agreement with his. The Hagins oscillogram obtained at 486 mμ and 12° C. shows a brief increase in absorption immediately after the flash, followed by an increase in transmission that is clearly composed of 2 rates; at 400 mμ following the flash there is a progressive increase in absorption, again consisting clearly of 2 rates. The rate constants of these 2 processes measured at 12° C. are 61.6 and 25.0 $sec.^{-1}$ and compare favorably with the 2 related constants 70.4 and 18.3 obtained by us from flashed rhodopsin solutions at 14.4° C. Although the data from the intact eye and solubilized rhodopsin can be compared only in part, the agreement that does exist suggests that the behavior of rhodopsin in solution is similar to the behavior of the pigment in intact retinal rods.

Relation to visual excitation. It is tempting to speculate on which of the transient events following flash illumination of rhodopsin are causally related to the electrical excitation of the photoreceptor. If time is the criterion employed, then any photochemical process having a duration shorter than the latent period of the photoreceptor's electrical response may be incriminated in visual excitation. Since latent periods of several milliseconds or more are common,[11] both the formation of photo-excited rhodopsin and the first two dark-decay processes may be incriminated (at 37° C).

References

1. Porter, G. 1950. Flash photolysis and spectroscopy. A new method for the study of free radical reactions. Proc. Roy. Soc. London. **A200**: 284–300.

2. Linschitz, H., V. J. Wulff, R. G. Adams, & E. W. Abrahamson. 1957. Light-initiated changes of rhodopsin in solution. Arch. Biochem. Biophys. **68:** 233–236.
3. Wald, G. & P. K. Brown. 1952. The role of sulfhydryl groups in the bleaching and synthesis of rhodopsin. J. Gen. Physiol. **35:** 797–821.
4. Hubbard, R. & A. Kropf. 1958. The effect of light on rhodopsin. Proc. Natl. Acad. Sci. **44:** 130–139.
5. Collins, F. D. & R. A. Morton. 1950. Studies in rhodopsin. III. Rhodopsin and transient orange. Biochem. J. **47:** 18–24.
6. Wald, G., J. Durell, & R. C. St. George. 1950. The light reaction in the bleaching of rhodopsin. Science. **111:** 179–181.
7. Lythgoe, R. J. 1937. The absorption spectra of visual purple and indicator yellow. J. Physiol. **89:** 331–337.
8. Broda, E. E. & C. F. Goodeve. 1941–1942. The behavior of visual purple at low temperature. Proc. Roy. Soc. London. **B130:** 217–225.
9. Hubbard, R. 1956. Geometrical isomerization of vitamin A, retinene and retinene oxime. J. Am. Chem. Soc. **78:** 4662–4667.
10. Hagins, W. A. 1956. Flash photolysis of rhodopsin in the retina. Nature. **177:** 989–990.
11. Wulff, V. J., W. J. Fry, & F. A. Linde. 1955. Retinal action potentials—theory and experimental results for grasshopper eyes. J. Cellular Comp. Physiol. **45:** 247–264.

KINETICS OF CONE PIGMENTS MEASURED OBJECTIVELY ON THE LIVING HUMAN FOVEA

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Introduction

When the human retina is viewed in an ophthalmoscope, the light entering the observer's eye has passed twice through the retina and hence has suffered absorption by the retinal pigments there. Since retinal pigments become nearly transparent upon photolysis, that is, "bleaching," the amount of pigment originally present can be measured by receiving the reflected light, not upon the observer's eye, but upon a photo cell, the output of which can be measured accurately. The increase in output after bleaching can be brought back to the initial value by interposing a suitable density in the light path, and the density that must be added will obviously be the same as the density of the pigment—for double passage—that has been bleached away.

When the region of the retina so examined is the parafovea, the chief pigment measured is rhodopsin. Previous publications by Rushton *et al.* (1955), Campbell and Rushton (1955), and Rushton (1956) describe the equipment and give rather strong evidence that rhodopsin density is what is measured. When the retinal region examined is restricted to the fovea, a visual pigment is also seen, but the pigment here is not rhodopsin; instead, it appears to be from the cones.

The kinetics of this supposed cone pigment forms the subject of this paper. The first step is to present some grounds for the belief that cone pigments can be measured in this way.

Method

Apparatus. This is similar to that already described for the measurement of rhodopsin by Rushton (1956 and 1957a), and is represented in FIGURE 1. It is constructed around a fine double monochromator designed by Stiles (1955), which was built by the National Physical Laboratory, Teddington, England. Light from a 6 v car headlight whose filament is focused upon the inlet slit I is dispersed by prisms, and an image of the spectrum is formed at QQ. If nothing obstructs the light here, it is all recomposed by the second pair of prisms and leaves by the exit slit E as white light. By means of slots with shutters in the plane QQ, wave bands may be selected as desired. The emerging light is brought by L_3 and L_4 to a focus upon the cornea of the subject's eye, the vertical image of the slit being 1 mm. nasal to the central point of the dilated pupil.

The subject thus sees L_4 in Maxwellian view, but the field is limited to 2° by stop S_1, which is fitted with cross hairs and placed at the focus of L_4, where it is seen sharply by the homatropinized (emmetropic) eye.

The ophthalmoscopic mirror M_4 reflects light from the temporal half of the pupil, and L_5 brings the retinal image to a focus in the plane of S_2, an iris stop

that accepts only the central 1.8° of the illuminated fovea when the subject fixates on the cross hairs. The retinal image may be directly viewed from O, or, by swinging the mirror M_5 into the position shown, the light is thrown onto the large cathode of an E.M.I. red-sensitive photomultiplier tube $P.C.$

Now, although a change in pigment density will produce a change in photocell output, we may not use the output to measure the density unless all other principal causes of output change have been eliminated. An important step in this direction is to compare the output from a light absorbed by the pigment with the output from deep red light of wave length 700 mμ. This is simply done by mounting at the inlet slit I a polaroid P_0 set in a ball race so that it can be rotated at 70 rps. Then, in the plane QQ a fixed polaroid

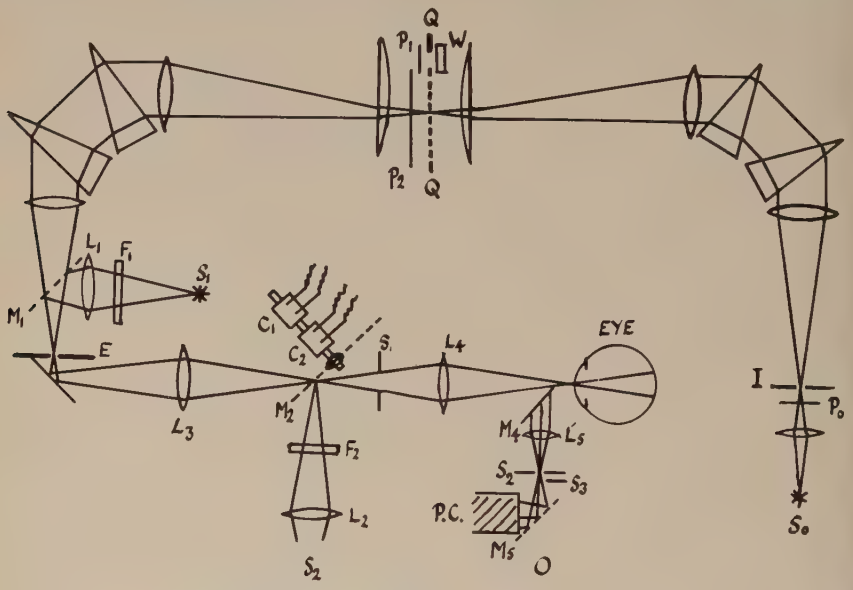

FIGURE 1. Reflection densitometer.

P_1 is placed in front of the slot in the deep red, where a photometric wedge W is also interposed, and a second polaroid P_2 is placed across the rest of the spectrum, with its axis 90° from P_1. In this way the output from the photocell will be

$$r \sin^2 pt + g \cos^2 pt \qquad (1)$$

where r is the output factor from the red light, g the output factor from green, t the time, and p the angular velocity of P_0. By moving W, the value of r may be changed and, when $r = g$, expression 1 is seen to become simply g while its AC component becomes zero.

The procedure is, therefore, to measure the AC output, to bring this to zero by moving W, and then to bleach the pigment. This will not affect the transmission of the deep red to which the pigment is transparent, but the change in transmission at the other wave length will cause an AC output

that requires a shift of W to restore it to zero. This change in W (wedge shift) is equal to the double density change of the pigment for the wave length used to measure it. This null method is seen to be unaffected by fluctuations in the brightness of the light source or by the gain of the photocell equipment. The measurements are made very much more precise by feeding the photocell output into a phase-sensitive rectifier operated by the pure sine wave from P_0. In this way all components of "noise" in the weak signal from the photocell are eliminated, except those that have the frequency and approximate phase of the true signal.

It is important to be able to measure the pigment level while bleaching is proceeding. The bleaching light is from source S_2, a headlamp bulb like S_0, suitably attenuated and colored if necessary by filters, and a neutral wedge at F_2. The filament is focused upon a mirror M_2 mounted on the shaft of a motor like two sails of a windmill. As the motor rotates at 8 rps the mirrors are interposed for one fourth of the cycle and, for the other three fourths, the passage is clear for light from the spectrometer. While the bleaching light from S_2 is shining upon the eye, commutators C_1 and C_2 on the motor shaft short-circuit the output from the photocell, and also stop the bleaching light from falling upon it by interposing a quickly moving shutter S_3 across the foveal image stop S_2. In this way the eye is more or less continuously bleached by the flickering flashes at 16 per sec. from S_2, and more or less continuously measured by the interleaved, balanced spectral lights that alone are admitted to the photocell, and that during more than half the cycle.

Procedure. The subjects were volunteers from the classes in physiology in Cambridge University. Their pupils were dilated with homatropine and the lens accommodation paralyzed. They were clamped into the apparatus by dental fixation and a molded forehead rest, and they fixed their gaze on the cross hairs continuously for periods of between 5 and 20 min., depending upon the nature of the experiment.

The reliability of the results depended entirely upon their steadfast cooperation, and their willingness to come repeatedly for a three-hour session of this exacting nature.

The Difference Spectrum in the Protanope and the Normal Eye

A protanope is a color-blind person who, like most color-blind people, cannot distinguish red from green. Protanopes are distinguished from deuteranopes, the other common class, by the fact that they cannot see far into the red end of the spectrum. To a protanope a deep red is practically black and, as a result, protanopes have usually been regarded as red-blind. If this means that the eye of the protanope has one less foveal pigment than the normal eye, it will be simpler to investigate. Willmer (1949) has found from psychophysical measurements that it appears to have a monochromatic fovea, and the objective measurements in my own studies substantiate this; hence the taking of measurements on the protanope eye is actually simpler, and is therefore considered first.

FIGURE 2 shows the effect on a protanope of a sudden (15 sec.) bleaching exposure followed by a period in the dark. After an initial dark-adaptation

period in dim light, measurements were taken as shown by the 3 circles at 0.155. Then a bright light was shone on the eye for 15 sec. and as soon as possible after its removal a new wedge setting was obtained to balance the altered photocell output. Subsequent measurements show a return to the initial value that takes about 8 min. Under these conditions the rhodopsin in the parafovea takes about 30 min. to be fully restored. This is discussed in greater detail by Rushton *et al.* (1955).

If the change measured in FIGURE 2 were due to the bleaching of a photosensitive pigment, one would expect to find a theoretical relationship between the energy of the bleaching flash and the amount of pigment bleached. This will be dealt with for the normal eye in the following section. Let it be said

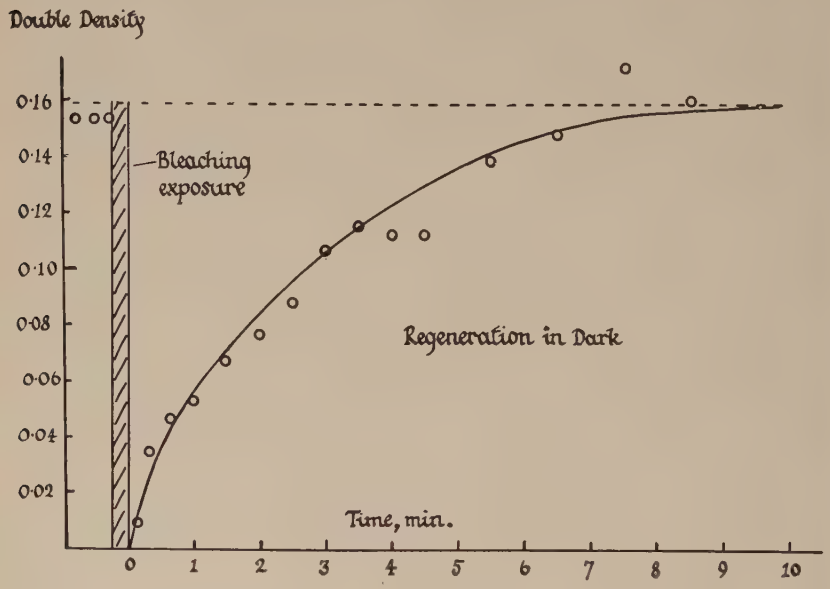

FIGURE 2. Change in foveal reflectivity of the protanope after 15-sec. bleaching, followed by 10 min. in the dark.

here simply that the same relationship has been found to be satisfied in the protanope, and thus the measurements of FIGURE 2 represent the density changes of one or more photosensitive pigments.

FIGURE 3 shows the measurements of the difference spectrum of two protanopes, that is, the change in density upon a full bleach when the measuring light used was of various wave lengths. The difference spectrum was found to be the same when the bleaching was partial and when the bleaching light was either bright orange or green. This points to the presence of only one pigment, and that conclusion has been confirmed by a more sensitive differential method. However, if only one photosensitive pigment is present and that is a *visual* pigment, its absorption spectrum should correspond to the spectral sensitivity curve, as has long been recognized for twilight

visibility and the rhodopsin spectrum. FIGURE 3 shows the well-known protanope visibility spectrum (Pitt, 1944), which substantiates the general reliability of the density measurements.

FIGURE 4 shows the results of difference spectra measured on the normal eye. Bleaching with a deep red light, which has no effect on the protanope at all, produces a difference spectrum in the normal eye having a maximum at

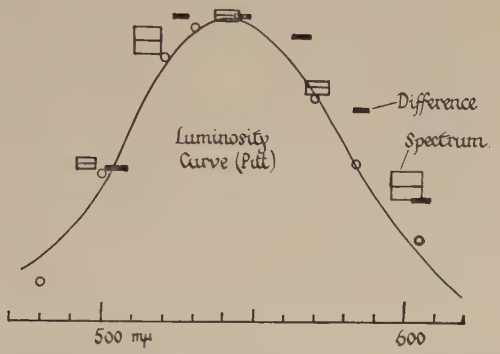

FIGURE 3. Difference spectrum of two protanopes. The rectangles give the difference spectrum of two subjects after full white bleach. The curve is Pitt's protanope luminosity, the circles are luminosity for one of these subjects.

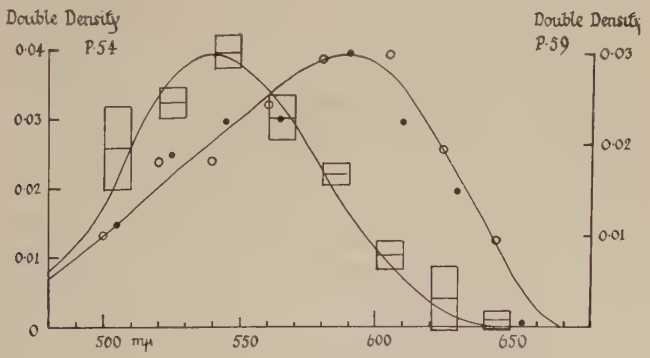

FIGURE 4. Difference spectrum of the two foveal pigments in the normal eye. The left curve is the same as that in FIGURE 3; the right curve was drawn free-hand.

about 590 mμ. A final bleach, after all this red-sensitive pigment has been bleached away, reveals a second pigment, which appears to be identical with that in the protanope. This is to be expected, since the protanope accepts normal color matches; hence, almost certainly, his pigments are normal pigments.

Measurements in the blue part of the spectrum are very hard to make, partly because the macular pigment greatly weakens the signals obtained. There is no evidence about the presence of a blue pigment on the fovea.

A preliminary note concerning these results in the normal eye has already appeared (Rushton, 1957b) and a more extensive preliminary discussion of the normal and the color-blind eye has also been presented (Rushton, 1957c).

Kinetics

There are two technical difficulties in the measurement of cone kinetics. First, the rate of bleaching is usually too fast to be followed by the wedge with sufficient accuracy. Second, the difficulties involved in measurements with one pigment are much increased from the presence of two. The second obstacle could be overcome by confining observations to protanopes, but valid results may be obtained from the normal eye using the following observations.

Preliminary measurements were made using the usual method of flicker between two wave lengths but, instead of one being in the far red and used as a control, wave lengths about 535 and 620 mμ were used. These were so adjusted that, upon bleaching with white light, there was just as much increase in transmissivity at one wave length as at the other; hence, upon total bleaching, the initial dark-adapted wedge setting was exactly unchanged. With this setting a red bleach caused a balance shift in one direction, and a blue-green bleach a shift in the other. However, a white bleach of various intensities never produced a change, nor was there any change during the 8-min. return to dark adaptation after a full bleach. It may be concluded, therefore, that the two pigments regenerate at the same rate and that white light bleaches both at the same rate—at least within the limits of our technique. Since, in the following investigation, all bleaching was done with white light, the two pigments may be treated as one.

Now the first experimental relationship we need is the fraction of pigment bleached by lights of various energy content when the exposure is too short for any appreciable regeneration to have taken place. It was judged that a 10-sec. exposure might not be too long, and this was confirmed by repeated bleaching measurements using, first, an intensity that bleached about half the pigment in 10 sec., and then exactly twice this intensity for a period of 5 sec. Measuring the wedge balance always exactly 15 sec. after extinction of the bleaching light resulted in the same change in both cases; thus, 10 sec. appears to be still within the Bunsen-Roscoe range, and regeneration is negligible.

Nevertheless, although measuring 15 sec. after the extinction of the bleaching light is valid for detecting a difference in bleaching in the two cases, it does not tell us what the bleaching was at the moment of extinction, but only what it was after 15 sec. of dark adaptation. Although this error is not great, it may be eliminated by the method shown in FIGURE 5. If after 10 sec. the bleaching light I_1 is not extinguished, the pigment level will continue to fall along the dotted line. If I_1 is extinguished, the level will rise along the dotted dark-adaptation curve. However, if I_1 is changed to a suitable value I_2, the regeneration rate can be exactly neutralized and the level will remain unchanged indefinitely. The experiment was to find, for each bleaching, the intensity I_2 that caused no change in level between the

first measurement, which was 15 sec. after the end of the 10-sec. exposure, and the last measurement, which was 90 sec. later. It was then assumed that the fraction x of pigment bleached would remain steady indefinitely, and had had that same value from the end of the 10-sec. bleach.

FIGURE 6 shows, plotted on an intensity scale of log trolands,* the experimental values of I_1 and I_2 that correspond to various fractions x of pigment bleached.

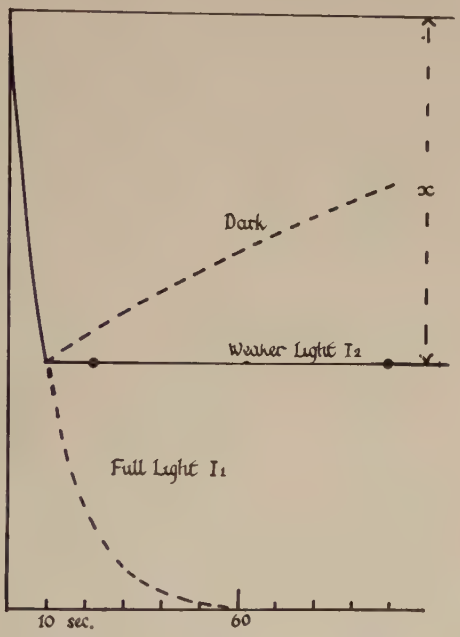

FIGURE 5. Illustration of 10-sec. bleach by I_1 with level x maintained by I_2.

The curve drawn through the points I_1 is the theoretical curve derived as follows.

If no regeneration occurs during the 10-sec. interval t_0 during which bleaching occurs, then

$$\frac{dx}{dt} = k(1 - x)I \tag{2}$$

where k is a constant.

$$\therefore \; - \log_e (1 - x) = kt_0 I$$

Let I_e be the value of I, such that, at the end of 10 sec. $(1 - x) = 1/e$. Then

$$1 = 10kI_e \tag{3}$$

or, in general,

$$- \log_e (1 - x) = I/I_e \tag{4}$$

* One troland (= one photon) is the retinal illumination produced by viewing a surface of luminance 0.1 mL (milli-Lambert) through a pupil 2 mm. in diameter.

Hence, if I_e is known, we may evaluate x for any intensity I from EQUATION 4, and the relation so found is the theoretical curve I_1 (FIGURE 6). If I_e is not known, the curve of FIGURE 6 may be slid horizontally, and this has been done to give the best fit with the points. The value of I_e thus obtained is 5×10^5 trolands.

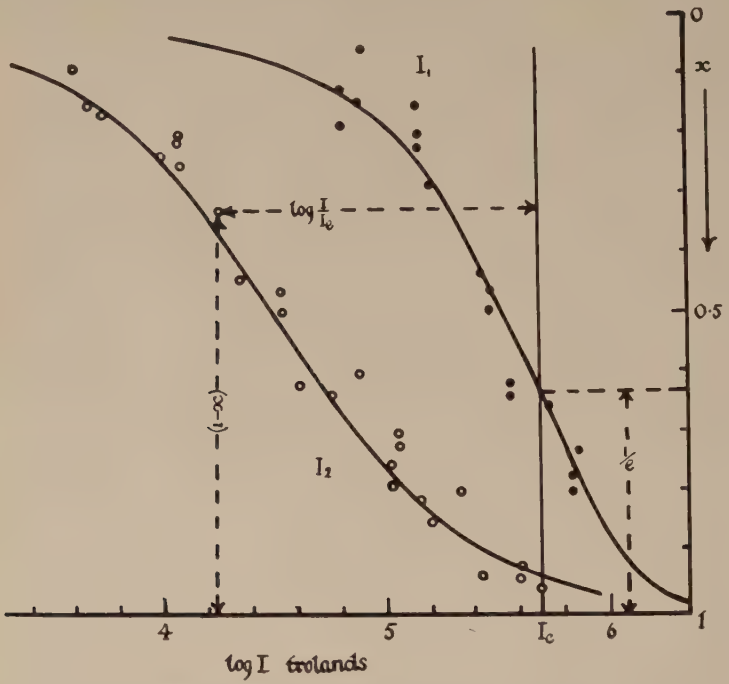

FIGURE 6. Experimental values of I_1 and I_2 that correspond to fractions x of pigment bleached. Dots relate the fraction x bleached (measured downwards) and log I_1, the intensity of the 10-sec. exposure. Circles relate x and log I_2, the maintained intensity. Curves are the theoretical expectations from EQUATION 6.

Inserting this in EQUATION 3, we obtain k, which, when substituted in EQUATION 2, gives

$$\frac{dx}{dt} = \frac{(1 - x)I}{5 \times 10^6} \tag{5}$$

Thus, in the steady state under illumination by I_2, the rate of regeneration must be exactly equal to the rate of bleaching; this is found by inserting the value of I_2 for I in EQUATION 5.

The circles in FIGURE 7 show for various levels of x the rate of regeneration in the light determined in this way. The rate is seen to be more or less proportional to x.

However, we may obtain an altogether independent measure of the regeneration rate at level x by performing the experiment illustrated in

FIGURE 2. The tangent drawn to the dark-regeneration curve at each level x will give the rate of regeneration at this level *in the dark*. These values are shown by the dots in FIGURE 7, obtained from an experiment on the subject who provided the circles, and at the same session. It appears that at any level x the rate of regeneration is $\frac{1}{130}$ per sec., whether in light or darkness.

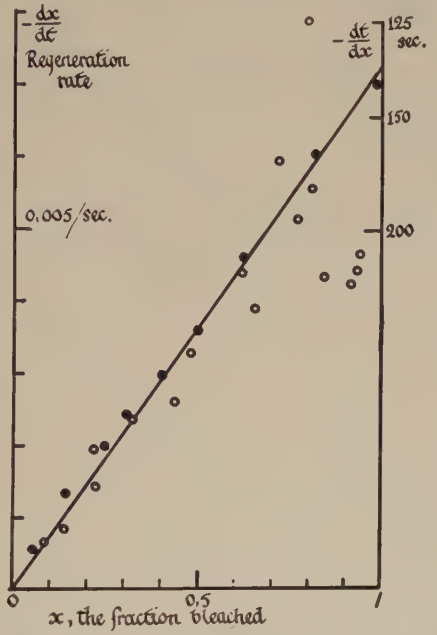

FIGURE 7. Rate of regeneration. Circles give for various fractions bleached, x, the rate of regeneration of cone pigments under steady illumination I_2 (FIGURE 6). Dots give the regeneration in the dark, obtained by drawing tangents to the curve through the dots of FIGURE 8.

We may thus extend EQUATION 5 to give the general equation for human cone kinetics

$$\frac{dx}{dt} = \frac{(1-x)I}{5 \times 10^6} - \frac{x}{130} \qquad (6)$$

where x is the fraction of pigment bleached, t is measured in seconds, and I is measured in trolands—white light—and is any known function of t.

FIGURE 8 gives an example of the application of EQUATION 6 to a simple pair of cases. Suppose that I is of intensity 4×10^4 trolands, and that it is turned on, (a) when the eye is fully dark-adapted, and (b) when the eye is fully bleached, and continues to shine indefinitely.

It is easy to see from EQUATION 6 that the intensity chosen is one that comes into equilibrium when $x = \frac{1}{2}$. Nor is it hard to derive that the time constant of adaptation should be half that of the dark-adaptation curve. The three curves of FIGURE 8 follow from EQUATION 6 without any arbitrary

feature. The dots show the dark-adaptation curve from which the dots in FIGURE 7 were obtained. The circles of FIGURE 8 show experimentally the bleaching or regeneration to equilibrium under I; it can be seen that the time constant is just about half that in dark adaptation, as predicted.

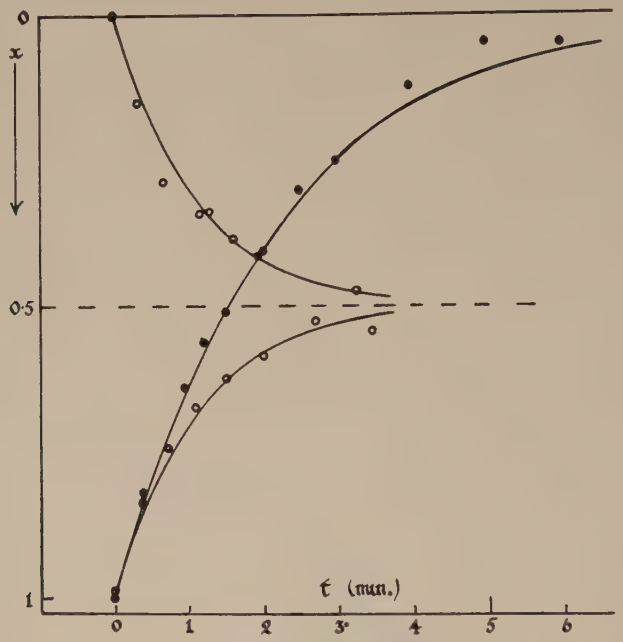

FIGURE 8. Example of the application of EQUATION 6 to a simple pair of cases. Dots give the dark regeneration curve, circles, the adaptation to a steady light of 4×10^4 trolands, starting either from full dark adaptation or full bleaching. Curves are the theoretical expectations from EQUATION 6 with no arbitrary constants.

The experimental points of FIGURE 8 adequately support the theoretical expectations.

Implications of Kinetics

Equilibrium levels. In equilibrium under a steady illumination of I trolands, the fraction x of pigment bleached from EQUATION 6 is given by

$$I = \frac{x}{1 - x} 4 \times 10^4 \qquad (7)$$

This is one form of Hecht's celebrated equation (1937), which has played so stimulating a part in promoting accurate experimental work in psychophysics and in attempts to relate sensory data with photochemical events.

As is well known, by application of this formula—or something very similar, Hecht was able to give accurate quantitative expression to the measurement of acuity, increment threshold, instantaneous threshold after light extinction, flicker, and other such data. His formula had one arbitrary

constant—the value of I that was necessary for half bleaching. In photopic conditions this was always somewhere between 10 and 100 trolands, but new techniques allow us to measure this value directly. It is seen in EQUATION 7 or in the plotted measurements of FIGURE 6 that the actual light required for half bleaching is around 4×10^4 trolands.

This is about 1000 times as great as is required for Hecht's interpretation; hence some other explanation is needed for the simple and connected relations that Hecht collected, verified, extended, and analyzed so cogently.

Photosensitivity. The photosensitivity ϕ is most easily defined by its reciprocal: $1/\phi$ = the number of quanta/cm.^{2} required to fall upon a pigment in order to leave only $1/e$ of it intact.

$$1/\phi = I_e t_0 = 5 \times 10^6 \text{ troland sec.}$$
$$= 4 \times 10^{15} \text{ quanta (555 m}\mu\text{) cm.}^{-2}$$

assuming 50 per cent transmission loss through the eye media, and parallel light through the pigment.

$$\therefore \ \phi = 2.5 \times 10^{-16} \text{ cm.}^2$$

Values between 2 and 6 have been found.

According to the Stiles-Crawford effect (1933) light that enters near the edge of the dilated pupil has a much reduced brightness. If this corresponds to a similar reduction in the bleaching of the pigment measured by reflection densitometry, the value of ϕ should be increased by about 50 per cent. Some further increase is due from the consideration that light cannot be regarded as being at the maximum wave length for both the foveal pigments at once.

Thus, the photosensitivity of the cone pigments is perhaps 7×10^{-16} cm.^{2}, which may be compared with 16×10^{-16} cm.^{2} found by Brindley (1955) for the red pigment, which he deduced from the alteration of color matches after adaptation to very bright lights. Dartnall *et al.* (1938) found that the photosensitivity of rhodopsin in solution was 0.9×10^{-16} cm.^{2}; hence the cones are considerably more photosensitive. Even allowing for molecular orientation, which would bring rhodopsin to 1.4×10^{-16}, the cones seem to be between 5 and 10 times as sensitive.

This is hardly likely to be a property of the pigment itself, but is rather the result of error in the assumption that light through the pigment was parallel. If both the light and the pigment were concentrated upon a very restricted region in the cone, it would explain the high photosensitivity and the high pigment density (about two thirds that of human rhodopsin) with a minute quantity extractable. Oblique incidence on the retina would cause the concentrated rays no longer to fall upon the most concentrated region of the pigment, and for this reason something like the Stiles-Crawford effect would result. Finally, the presence of the dense pigment particle at the focal point of the cone-lens system would result in a very great magnification of the particle when viewed from the vitreal aspect, as in reflection densitometry. It is probable that the whole inner segment of the cone appears filled with

this dense pigment and so permits the very small *quantity* that is actually there to be measured with a certain precision.

The biochemistry of regeneration. The most outstanding achievement in relation to the regeneration of cone pigments is the synthesis of iodopsin *in vitro* by Wald *et al.* (1955). They used opsins from the eyes of fowls and, although the proteins from the cones and the rods were not separated, there was no confusion of results, since the synthesis of iodopsin occurred 500 times as rapidly as that of rhodopsin and was complete, while that of rhodopsin had hardly begun.

Under the conditions of the experiment *in vitro*, a fair amount of 11-*cis* retinene was supplied initially and, as the reaction proceeded, this was partly used up and no more was added. The reaction may be represented:

$$\underset{x}{\text{opsin}} + \underset{y}{\text{retinene}} \rightarrow \text{iodopsin} \tag{8}$$

If x is the amount of opsin present at any time t, and y is the amount of retinene, these investigators found that the synthesis proceeded according to a second-order reaction (EQUATION 8) that gives

$$\frac{dx}{dt} = -k_1xy \tag{9}$$

The retinene concentration y was expressed in units of "extinction" and, at 10° C., k_1 was found to be 0.16 per sec. per extinction unit.

Now in the living human eye at 37°C., regeneration, whether in light or dark, was found (FIGURE 7) to be

$$\frac{dx}{dt} = -\frac{x}{130}$$

Thus, if we may apply EQUATION 9 to the living eye,

$$y = \frac{1}{130k_1} \tag{10}$$

So the retinene concentration keeps at a constant and very low value, and the synthesis degenerates into a first-order reaction.

This low constant retinene concentration follows from the rapid reduction of retinene to vitamin A, which was already foreshadowed by the observations of Kühne (1877) and has been worked out in greater detail by Wald and Hubbard (1948), Bliss (1949 and 1951), and Wald (1951).

$$\text{Vit. A ester} \leftarrow \text{Vit. A} \underset{\text{DPN-H}_2}{\overset{\text{DPN}}{\rightleftharpoons}} \text{retinene} \xrightarrow{\text{opsin}} \text{iodopsin} \tag{11}$$

The rapid reduction of retinene to the vitamin by means of alcohol dehydrogenase results in a "buffered" concentration of retinene, low in amount, but rapidly replaced and so steadily maintained.

Its level might be found from EQUATION 10 if we assume that k_1 for the fowl's photopsin is the same as that for man, and that at 37° C. the value of k_1

is 16 times what it is at 10° C. The retinene level then amounts to 0.003 of an "extinction unit."

Wald *et al.* (1957) have observed that the rod opsins of frogs and alligators are responsible for a variation that is about twentyfold in the relative rates of rhodopsin synthesis. It may be unsound, then, to suppose that the fowl's photopsin has the same k_1 as man. This supposition is supported, however, by observations on the dark-adaptation curves of fowls, in particular the very pleasing measurements by Ratliff and Blough (1954) with the pigeon, in which both cones and rods were found to have a time course of adaptation very similar to that of man.

In any case, the kinetics of EQUATION 11 would lead to a buffered low-constant level of retinene that would reduce iodopsin regeneration to a first-order process in the eye. It is therefore satisfactory that this is the result found by the two methods of measurement plotted in FIGURE 7, each of which falls along the same straight line through 0.

Summary

A brief account is given of the method of reflection densitometry by which the density of pigments can be physically measured in the living eye. Measurements made upon the fovea of a protanope, the red-blind dichromat, show that the pigment is bleached in bright light and regenerates in the dark, the latter process taking about 8 min. for completion. The pigment bleached has a difference spectrum with a maximum at 540 mμ, and, since this is the same upon partial bleaching by orange or green light, it appears to be a single pigment. If this protanope pigment is that upon which the eye's sense of brightness depends, its absorption spectrum should coincide with the protanope visibility. FIGURE 3 shows a fair agreement between the difference spectrum and the visibility curve.

The normal fovea contains two pigments. One is the protanope pigment, the other is more red-sensitive with a maximum at 590 mμ. White lights are found to bleach both normal pigments at equal rates and the normal pigments also regenerate at equal rates; thus, with white light, they may be treated as a single pigment.

Two relations were determined: (1) what fraction x of pigment is bleached by a 10-sec. exposure to various intensities I_1, and (2) what value of x comes into equilibrium with a continued exposure I_2. Relation 1 satisfies the theoretical expectation for the bleaching of a photosensitive pigment. The photosensitivity is probably about 7×10^{-16} cm.^{2} Relation 2 shows that bleaching is opposed by regeneration in the light at a rate of $x/130$ per sec. The curve of regeneration in the dark gives the same values. EQUATION 6 gives x, the fraction bleached under any fluctuating light $I(t)$, and FIGURE 8 shows curves calculated *without any arbitrary constants*, as well as the degree to which observations correspond. The intensity for half-bleaching, which is required by Hecht's photochemical equation as applied to acuity, and other such sensory data, is actually too small by a factor of about 1000.

Retinene is known to be in equilibrium with vitamin A, the reaction being very fast in the direction of reduction. This will keep the retinene at a low

but constant level and will make cone pigments regenerate at a speed proportional to the concentration of opsin, x. This accords with the above observations that bleaching is opposed by regeneration in the light or in the dark at a rate of $x/130$ per sec.

Acknowledgments

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References

Bliss, A. F. 1949. Reversible enzymic reduction of retinene to vitamin A. Biol. Bull. **97:** 221.

Bliss, A. F. 1951. The equilibrium between vitamin A alcohol and aldehyde in the presence of alcohol dehydrogenase. Arch. Biochem. Biophys. **31:** 197.

Brindley, G. S. 1955. A photochemical reaction in the human retina. Proc. Phys. Soc. London. **B68:** 862–870.

Campbell, F. W. & W. A. H. Rushton. 1955. Measurement of the scotopic pigment in the living human eye. J. Physiol. **130:** 131–147.

Dartnall, H. J. A., C. F. Goodeve & R. J. Lythgoe. 1938. The effect of temperature on the photochemical bleaching of visual purple solutions. Proc. Roy. Soc. London. **A164:** 216–230.

Hecht, S. 1937. Rods, cones, and the chemical basis of vision. Physiol. Revs. **17:** 239.

Kühne, W. 1877. Über den Sehpurpur. Untersuch. physiol. Inst. Heidelberg. **1:** 15–103.

Pitt, F. H. G. 1944. The nature of normal trichromatic and dichromatic vision. Proc. Roy. Soc. London. **B132:** 101–118.

Ratliff, F. & D. S. Blough. 1954. Behavioral studies of visual processes in the pigeon. Technol. Rept. Psychol. Lab. Harvard Univ.

Rushton, W. A. H. 1956. The difference spectrum and the photosensitivity of rhodopsin in the living human eye. J. Physiol. **134:** 11–29.

Rushton, W. A. H. 1957a. Blue light and the regeneration of human rhodopsin *in situ*. J. Gen. Physiol. **41:** 419–428.

Rushton, W. A. H. 1957b. Physical measurement of cone pigment in the living human eye. Nature. **179:** 571–573.

Rushton, W. A. H. 1957c. Human cone pigments. Paper No. 1. Natl. Physics Lab. Symposium. Teddington, England.

Rushton, W. A. H., F. W. Campbell, W. A. Hagins & G. S. Brindley. 1955. The bleaching and regeneration of rhodopsin in the living eye of the albino rabbit and of man. Optica Acta **1:** 183–190.

Stiles, W. A. 1955. The basic data of colour matching. 18th Thomas Young Oration. Phys. Soc. Year Book. **:** 44–65.

Stiles, W. S. & B. H. Crawford. 1933. The luminous efficiency of rays entering the pupil at different points. Proc. Roy. Soc. London. **B112:** 428–450.

Wald, G. 1951. The chemistry of rod vision. Science. **111:** 179.

Wald, G., P. K. Brown & D. Kennedy. 1957. The visual system of the alligator. J. Gen. Physiol. **40:** 703–713.

Wald, G., P. K. Brown & P. H. Smith. 1955. Iodopsin. J. Gen. Physiol. **38:** 623–681.

Wald, G. & R. Hubbard. 1948–1949. The reduction of retinene to vitamin A *in vitro*. J. Gen. Physiol. **32:** 367.

Willmer, E. N. 1949. Further observations on the properties of the central fovea in colour blind and normal subjects. J. Physiol. **110:** 422–446.

MODELS OF THE VISUAL PROCESS

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Questions Still To Be Answered

It seems to an outsider such as myself that the more closely the photochemistry of vision is examined, the more complicated the field appears to be. One might assume that the action of light on the retina would have its counterpart in the photochemistry of purely synthetic systems and that the visual process is not incompatible with the known laws of physical chemistry.

The first question a photochemist might ask is: "What is the chemical nature of the chromophore of rhodopsin?" Since a product of the photobleaching of rhodopsin is retinene,[1] that is, vitamin A aldehyde,[2] it is natural to assume that the chromophore is a dimer of the C_{20} conjugated system with conjugation preserved in the dimer.[3] Compounds of this type—for example, β-carotene, a provitamin A—absorb maximally near 500 mμ, as does rhodopsin, but in solution or in the colloidal state they exhibit other maxima that do not appear in the rhodopsin spectrum. Calculations have been presented indicating that one molecule of rhodopsin possesses only one C_{20} chromophore.[4] Apart from these arguments one would expect that if the chromophore were β-carotene it would be readily extractable from its protein substrate, opsin, by means of a hydrocarbon solvent. In our laboratory we have failed to find any photochemical activity of colloidal β-carotene, despite the report of Karrer and Strauss[5] to the contrary. We attribute their apparent acceleration of the autoxidation of benzidine by colloidal β-carotene as the photosensitizer to failure to remove heat and ultraviolet light from their incident light.

Several arguments have been presented that attribute the color of the chromophore to some special interaction of vitamin A aldehyde with the protein substrate.[6] Extended conjugation between retinene and opsin would require some special structure in the protein that is not evident in its spectrum. The ultraviolet spectrum of opsin reveals only the presence of tyrosyl and phenylalanyl residues, and no additional conjugated structures. Although ionic Schiff bases could arise from interaction of aldehyde of retinene with amino groups of the protein, this would not produce the required 120 mμ shift in spectra; at least, that has been our experience in the laboratory with model aldehydic compounds. Furthermore, the Schiff bases made with retinene do not bleach with light. If hemiacetals were formed[7] no appreciable spectral shift should result. Furthermore, I find it difficult to visualize how the sulfhydryl groups of the protein would make themselves available merely by the photoisomerization of a chromophore that is attached to the protein.[7]

I do not pretend to know the answers to these vexing questions. However, I shall call your attention to some studies I have made on the photochemical properties of some purely synthetic pigments. My findings may stimulate

your thinking along different lines than those heretofore pursued to explain the photochemical aspects of vision.

Photoreduction

It has been known for many years that in the presence of mild reducing agents, certain dyes rapidly revert to their leuko or colorless form when illuminated by visible light.[8-10] The addition of electrons and/or hydrogen atoms interrupts the long conjugated system of the dye so that it is not light-absorbing above 400 mμ. The reduction does not proceed in the dark because the reducing agent is so chosen that such a reaction is thermodynamically impossible. In fact, for certain dyes such as the acridines the reduction can proceed in the dark only if the most powerful reducing agents such as sodium amalgam are employed whereas, in the light, the reduction proceeds with a quantum yield of unity in the presence of very mild reducing agents. In this case[11] the photoreduced dye is now a powerful reducing agent and can reduce the oxidized reducing agent and re-form the dye in the dark. By employing acriflavine, one can convert light into electricity efficiently, the potential produced being about one volt. The photoreduced dye can also reduce other substances, such as nitrobenzene, to aniline and certain aldehydes to their alcohols. The reduced dye readily reacts with oxygen to produce free radicals, possibly OH, which form hydrogen peroxide. If a vinyl monomer is present, polymerization will ensue,[12] and quantum yields for the conversion of monomer run as high as one billion.[13]

Certain dyes, such as the porphyrins, can be photoreduced in stages, as can be seen by their slight spectral shifts, and each species is capable of being further photoreduced. We have also found that intermediate states of reduction of the thiazine dyes can be trapped in glasses, for example, glucose glass, which serve as hydrogen donors.[14]

There is a wide variety of electron donors for photoreduction of dyes. Among these are the biologically important reducing agents, glutathione, ascorbic acid, glucose, and cysteine. Metal chelating agents containing secondary or tertiary nitrogens also serve as electron donors for photo-excited dyes.[15] Such substances are not reducing agents in the ordinary sense, in that they are difficult to oxidize by the usual oxidizing agents.

The chemically reactive excited state of the dye molecule is a long-lived state with a lifetime, in water, about 10^5 greater than that of the first singly excited state, which has a lifetime of about 10^{-9} sec.[16, 17] The sensitizing dye in photooxidation, which is sometimes referred to as the photodynamic effect, also goes through a long-lived excited state. The dye in this state readily reacts with oxygen, and the photoperoxide produced oxidizes the substrate with regeneration of the dye. We have found that only those dyes capable of being photoreduced can serve as sensitizers of photooxidations.[18]

Dyes in the Bound State

Many dyes exhibit dramatic differences in their properties when they are bound to high polymeric substances as compared with their properties in the unbound state. For example, the basic triphenylmethane dyes are readily

reduced, in the dark, by sodium bisulfite, yet they staunchly resist reduction, even with very powerful reducing agents, when the dyes are bound to a polymeric acid.[19] In the light the situation is reversed; here the bound dye is easily photoreduced in the presence of mild reducing agents, while the free dye is not.[19] Similarly, acriflavine in the presence of ascorbic acid at *p*H 7 is more readily photoreduced if the dye is bound to desoxyribonucleic acid than when it is free.[20]

Frequently binding of the dye results in a shift in absorption maximum of about 10 mμ to longer wave lengths. The fluorescence may increase or decrease on binding. With acriflavine the fluorescence is quenched by trace amounts of nucleic acid,[20] while with auramine fluorescence appears only if the dye is bound. This latter dye is one of many having "floppy" structures, that is, structures that allow for internal rotation, as in diphenylmethane, triphenylmethane, and stilbene derivatives. None of these dyes fluoresce in aqueous solutions, but they fluoresce strongly if they are bound to high polymers or are in media of high viscosity.[21] Planarity of the dye molecule seems to be a requirement for fluorescence, and it has been shown[21] that fluorescence will not occur if the groups move by rotational diffusion out of the plane by more than two degrees during the short lifetime, about 10^{-9} sec., of the singlet excited state.

Binding of a dye to some high polymeric substrate is enhanced if a dye capable of *cis-trans* isomerism is in the *trans* configuration. Thus chrysophenine G in the *trans* configuration binds far more effectively to cotton than does the photoproduced *cis* form.[22] Conversely, we have indications that such dyes undergo photoisomerism less readily in the bound state than they do when free in solution.

Photochemical Properties of Thiocarbonyls

During the course of our studies on the mechanism of cleavage of disulfides, N. A. Rosenthal and I noticed that alkali attack on unsymmetrical disulfides led to fleeting blue- and red-colored intermediate compounds.[23] The blue compound is a thiocarbonyl, and we postulated the formation of such groups as intermediates in the alkaline cleavage of any disulfide.[24] Thiobenzophenone, for example, is a fairly stable blue compound with absolute maximum at 595 mμ; it is made most conveniently[25] by passing H_2S through an acidified solution of benzophenone, which has absolute maximum at 250 mμ. Thioaldehydes are also colored, but have not been isolated because of their tendency to trimerize almost immediately on formation,[26] although we have had some success in stabilizing them in viscous media. The physical basis for the enormous shift of absorption maximum to longer wave lengths on merely replacing an oxygen atom by sulfur has been considered by molecular spectroscopists.[27, 28] We are inclined to attribute the effect to expansion of the outer vacant *d* orbitals of sulfur by accepting electrons from other portions of the molecule to form highly directed π bonds.[29]

In the course of our examination of thiobenzophenone, L. Citarel and I have recently discovered that alkaline solutions of this compound, and some derivatives, are highly sensitive to light. Red light converts the blue com-

pound to a red pigment. When illuminated with green light, the red pigment is converted into a yellow pigment. The yellow pigment is convertible into benzophenone, which is colorless. Sulfhydryl groups are produced somewhere in the process.

The red species may be somewhat stabilized by carrying out the photochemical reaction in glycerol, although it is converted back to the blue form by cooling the red glycerol solution in the dark. The blue dye bound to Nylon is converted by white light to the red species, but under these conditions the red form is very stable toward light. Only the yellow form appears on irradiation with white light when using six other polymer substrates. Thiobenzophenone binds very strongly to wool, but still fades with light. The red form on Nylon is an acid-base indicator in the dark, and becomes yellow with acid, which then reverts to red with alkali.

Although the mysteries of these reactions have yet to be understood, the results do suggest a new approach to the chemistry of the visual pigments. Suppose that the pigment of rhodopsin is thio vitamin A aldehyde, which is stabilized by binding to opsin. Then, by analogy with thiobenzophenone, the compound should be colored. It should be light-sensitive and give colored intermediates that could be trapped in viscous media[30] and that are acid-base indicators.[31, 32] Sulfhydryl groups would be produced in the photochemical reaction,[7] and a final product would be vitamin A aldehyde. The mode of bonding of the original pigment to opsin could be through van der Waal's forces, especially if lipid material is present, since thioretinene should have a very great polarizability. This binding is mainly of the hydrogen-bonding type when the sulfur is replaced with oxygen. Thio groups do not participate in hydrogen bonding as do carbonyl groups.

Attempts have been made to synthesize thio vitamin A aldehyde in order to test my hypothesis directly. However, in the course of acid treatment, a solution of vitamin A aldehyde turns various colors, which are possibly related to the carbonium ion intermediates postulated in the synthesis of anhydro vitamin A (axerophtene) by treating vitamin A with strong acid.[33] Incidentally, these colored ionic forms do not bleach with light. Obviously, for the synthesis of thio vitamin A aldehyde another route that avoids the use of strong acid must be devised.

References

1. Wald, G. 1935. J. Gen. Physiol. **19**: 351.
2. Ball, S., T. W. Goodwin & R. A. Morton. 1948. Biochem. J. **42**: 516.
3. Wald, G. 1949. Document. Ophthal. **3**: 94.
4. Hubbard, R. 1954. J. Gen. Physiol. **37**: 381.
5. Karrer, P. & W. Strauss. 1938. Helv. Chim. Acta. **21**: 1624.
6. Dartnall, H. J. A. 1957. The Visual Pigments. Methuen & Co., Ltd. London, England.
7. Wald, G. & P. K. Brown. 1951–1952. J. Gen. Physiol. **35**: 797.
8. Mudrovcic, M. 1928. Z. wiss. Phot. **26**: 171.
9. Weber, K. 1931. Z. physik. Chem. **B15**: 18.
10. Holst, G. 1934. Z. physik. Chem. **B169**: 9.
11. Oster, G. & F. Millich. 1958. To be published.
12. Oster, G. 1954. Nature. **173**: 300.

13. Oster, G. K., G. Oster & G. Prati. 1957. J. Am. Chem. Soc. **79**: 595.
14. Oster, G., B. Broyde & J. Dubien. 1958. To be published.
15. Oster, G. & N. Wotherspoon. 1957. J. Am. Chem. Soc. **79**: 4836.
16. Oster, G. & A. H. Adelman. 1956. J. Am. Chem. Soc. **78**: 913.
17. Adelman, A. H. & G. Oster. 1956. J. Am. Chem. Soc. **78**: 3977.
18. Oster, G. & R. Kimball. 1958. To be published.
19. Oster, G. & J. S. Bellin. 1957. J. Am. Chem. Soc. **78**: 294.
20. Oster, G. 1951. Trans. Faraday Soc. **47**: 660.
21. Oster, G. & Y. Nishijima. 1956. J. Am. Chem. Soc. **78**: 1581.
22. Atherton, E. & R. H. Peters. 1956. Chem. Soc. London. Spec. Publ. No. **4**: 22.
23. Rosenthal, N. A. 1955. Studies on the Disulfide Bond. Ph.D. Thesis. Polytechnic Institute of Brooklyn. Brooklyn, N. Y.
24. Rosenthal, N. A. & G. Oster. 1954. J. Soc. Cosmetic Chemists. **5**: 286.
25. Staudinger, H. & H. Freudenberg. 1928. Ber. **61**: 1576.
26. Migrdichian, V. 1957. Organic Synthesis. **I.** Reinhold. New York, N. Y.
27. Lewis, G. N. & M. Kasha. 1945. J. Am. Chem. Soc. **67**: 994.
28. McConnell, H. 1952. J. Chem. Phys. **20**: 700.
29. Craig, D. P., N. Maccoll, R. S. Nyholm, L. E. Orgel & L. E. Sutton. 1954. J. Chem. Soc. : 332.
30. Wald, G., J. Durell & R. C. C. St. George. 1950. Science. **111**: 179.
31. Chase, A. M. 1936. J. Gen. Physiol. **19**: 577.
32. Lythgoe, R. J. 1937. J. Physiol. **89**: 331.
33. Meunier, P. & A. Vinet. 1947. Chromatographie et Mésomérie. : 74. Masson & Cie. Paris, France.

ENERGY TRANSFER IN ORDERED AND UNORDERED PHOTOCHEMICAL SYSTEMS*

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Introduction

The phenomenon of energy transfer has been receiving an ever-increasing amount of attention from physicists, physical chemists, and biochemists since the pioneering work of Franck and Cario,[1] Franck and Teller,[2] and Vavilov.[3] This concept has proved to be of fundamental importance for an understanding of many of the photoinduced phenomena of molecules, both in solution and in the solid state, and is proving to be of increasing significance to biology.

Our concern here is mainly with a qualitative discussion of the theoretical aspects of energy migration, with some of the experimental criteria of this phenomenon and, finally, with its possible role in the primary quantum-conversion act in photosynthesis.

General Considerations of Energy Transfer in Unordered Systems

Spectroscopic properties of molecules in solution. Some of the main qualitative features of the effects of visible and U.V. radiation on molecules in solution may be understood by a consideration of FIGURE 1. Process *a* represents the absorption of a quantum of light by the molecule, resulting in a change in its electronic state. Molecules in the lowest excited singlet state may then undergo one of four processes: they may emit a quantum of light as fluorescence (process *b*); the electronic excitation energy may be degraded into heat (process *c*); a small portion of the electronic energy may be degraded into heat, concomitant with an unpairing of electron spins, resulting in an intersystem crossing into the lowest excited triplet state (process *d*); or the quantum of excitation energy may be transferred to another molecule. Molecules in the lowest excited triplet state may similarly undergo one of three processes: phosphorescence (process *f*), thermal degradation (process *e*), or energy transfer to another molecule.

Theoretical aspects of energy transfer. There are three mechanisms by which electronic excitation energy may be transferred from one molecule to another in unordered systems, namely:

(1) The emission of a quantum of radiation by the excited molecule followed by the reabsorption of this quantum by an unexcited molecule. This may be repeated many times. The probability of this process is determined simply by the Beer-Lambert law and by the geometry of the system. In general, the lifetime of the excited state of a particular molecule remains

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† National Science Foundation Postdoctoral Fellow, 1956–1957.

the same, but the lifetime of the emission in a finite system may be increased by the "imprisonment of radiation."[4] This mechanism has been shown to be of relatively minor importance in energy transfer in solution.[5, 6]

(2) The transfer of electronic excitation energy through close collisions between excited and unexcited molecules. The energy levels of the molecules are, in general, significantly perturbed by such collisions and, in this way, the absorption and emission spectra of the components may be changed. If, on occasion, only a small amount of the excitation energy is removed—and is transformed into vibrational energy of the acceptor molecule—the excited molecule may be brought into the triplet state.[7] Such a process may occur with particularly high probability if the acceptor molecule contains an atom of high atomic number or is paramagnetic.[8, 9] The close-collision mechanism

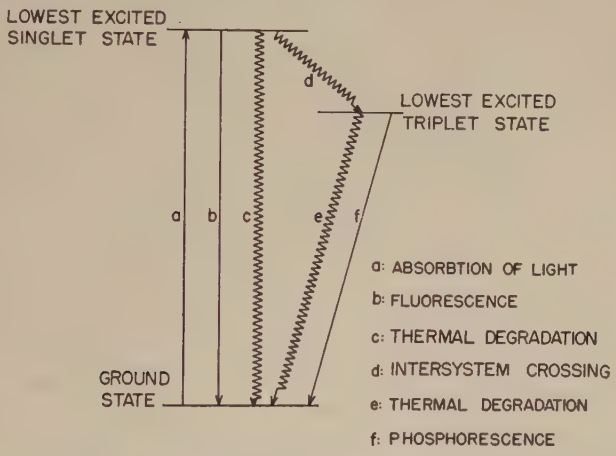

FIGURE 1. Lowest electronic energy levels of an isolated molecule. Straight lines represent radiative processes; zigzag lines represent radiationless (thermal) processes.

is believed to be the most important one in the fluorescence of liquid organic solutions induced by high-energy radiation.[6] It has been shown to be unimportant in some U.V.-induced energy-transfer phenomena.[10]

(3) The transfer of electronic excitation energy through collisions over a distance of several molecular diameters (resonance transfer).[11-14] The main quantitative theory of resonance transfer, which is due to Förster,[14] is based on a calculation of a mutually induced dipole interaction between donor and acceptor molecules, both of which are capable of being excited to the same energy level. The theory predicts that the probability of transfer is proportional to the extent of the overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, and also to the intensity of these transitions. This phenomenon may be thought of as analogous to the property of resonance in organic molecules inasmuch as, during the actual collision, the interaction between the molecules makes it impossible to consider the excitation energy as belonging to only one of the partners; rather, it must be thought of as belonging to both of them simultaneously. Thus, on

subsequent separation of the colliding molecules, there is a definite calculable probability that the energy will be found in the previously unexcited molecule. Förster estimated that, for typical dye molecules, that is, molecules with intense transitions in the visible region, the probability of energy transfer during an excited-state lifetime of 10^{-8} sec. becomes equal to the probability of fluorescence when the colliding molecules come within about 100 Å of each other—that is, about 10 times their ordinary kinetic-collision diameter. The probability of transfer, and thus the number of molecules over which transfer occurs, is also inversely proportional to the sixth power of the distance between the molecules and therefore directly proportional to the square of the concentration. In general, the absorption and emission spectra of the components are not changed by resonance transfer. This mechanism is generally considered to be the most important one in phenomena of energy transfer induced by visible or U.V. light.

Experimental aspects of energy transfer. Efficient energy transfer has been demonstrated thus far only from aromatic compounds, with one exception, 1,4-dioxane.[5] Energy transfer from many other types of organic molecules, both saturated and unsaturated, has been observed, but only with lower efficiency. This is probably because the excited-state lifetimes of these molecules are considerably shorter than those of the aromatic solvents. For this reason a higher concentration of acceptor molecules is necessary. With such poor solvents it is possible to increase the yield of energy transfer by adding a small amount, perhaps 10 per cent, of an efficient energy acceptor. This acceptor then mediates the transfer of energy from solvent to solute.

The general types of observable phenomena that can be interpreted in terms of energy transfer are as follows.

(1) Sensitized emission: in such measurements one dissolves a small amount of an emitting substance in a solvent that absorbs energy at somewhat shorter wave lengths (that is, higher energy) than does the solute. Upon excitation of such a system with radiation absorbed only by the solvent, the resulting emitted light is that characteristic of the solute. The solvent emission is normally almost completely absent.

(2) Concentration depolarization: the fluorescence emitted by dilute solutions of organic molecules in viscous solvents is normally partially polarized, owing to a limited amount of orientation of the molecules. As the concentration is increased, however, the extent of polarization decreases. This is due to a transfer of excitation energy between molecules in different orientations.

(3) Self-quenching of fluorescence: the quantum yield of fluorescence of solutions of organic molecules generally decreases with increasing concentration. This phenomenon is made somewhat more complicated by the possible occurrence of other effects such as the equilibrium formation of relatively stable nonfluorescent dimers.[15] In many cases, both the nature of the concentration dependence and the effects of temperature and viscosity enable one to decide between the mechanisms. If resonance transfer is to lead to self-quenching, some of the molecules must be in a nonfluorescent state and, furthermore, the lifetime of such a state must be comparable to,

or longer than, the average time that the excitation energy spends in any one molecule. Förster suggests that such a nonfluorescent energy sink is a statistical dimer.[14]

(4) Quenching of fluorescence by solutes: this phenomenon is essentially similar to self-quenching in that the final energy acceptor must be nonfluorescent, the electronic excitation energy eventually being degraded into the thermal energy of the solvent. Interpretations in terms of resonance transfer may be complicated by the occurrence of intermolecular spin-orbital perturbations[9] leading to an increased probability of an intersystem crossing into the triplet state.

It would be impossible here to review all the many systems in which the above phenomena have been observed. However, we shall mention a few of the more significant examples. Förster,[16] in a study of the concentration dependence of the quenching of the fluorescence of solutions of tryptaflavine by rhodamine B, demonstrated that nonradiative energy transfer occurs efficiently at distances as great as 70 Å, in agreement with the predictions of his theory. Similarly, Lavorel,[17] in a study of alkaline fluorescein solutions, was able to calculate that a resonance transfer of energy occurred over an average of about 300 molecules. Of particular biological significance is the demonstration by Watson and Livingston[18] and by Duysens[19] of the sensitization of chlorophyll *a* fluorescence in methanol solution by chlorophyll *b*. In addition, there have been a number of studies of energy transfer in protein-dye conjugates[20] whereby energy absorbed in the protein portion of the conjugate (for example, lysozyme, bovine plasma albumin, chymotrypsinogen, and ribonuclease) excited the fluorescence of the dye (for example, 1-dimethylaminonaphthalene-5-sulfonyl chloride).

A series of very interesting experiments by Terenin and Ermolaev[21-23] has demonstrated sensitized phosphorescence in rigid solutions at −180° C. These workers studied various combinations of naphthalene, benzaldehyde, biphenyl, and benzophenone. A donor molecule was selected that would have its lowest excited singlet state below that of the acceptor and its lowest triplet above that of the acceptor. When such mixtures were illuminated with light absorbed only by the donor, the phosphorescence of the acceptor was sensitized and that of the donor quenched. Terenin and Ermolaev interpreted these results in terms of an energy transfer between the triplet states of the molecules involved.

Whereas the application to biology of the concepts outlined above must be reserved mainly for the future, one might cite a number of examples in which energy transfer is of importance. The first, of somewhat trivial significance, is scintillation counting,[24] in which solutions of hydrocarbons are used to detect and measure high-energy radiations in tracer work. The second is the well-known demonstration of the transfer of energy from various plant pigments such as phycocyanin and phycoerythrin to chlorophyll in plant material.[25, 26] Finally, we might mention the experiments of Arnold and Meek,[27] who have demonstrated the transfer of energy among chlorophyll molecules in the grana through a study of the polarization of the fluorescence. Rabinowitch[28] has suggested that this energy migration is a

result of resonance transfer and has estimated that, for excited-state lifetimes of about 10^{-9} sec. and intermolecular distances between chlorophyll molecules of about 10 Å, energy-transfer chains of the order of 100 or 1000 molecules could easily occur.

General Considerations of Energy Transfer in Ordered Systems

Theoretical aspects. There are three mechanisms by which energy transfer may proceed in ordered systems:

(1) The emission of a quantum of radiation, followed by its reabsorption by an unexcited molecule. This is exactly analogous to the mechanism proposed for fluid solutions. The main proponent of this theory has been

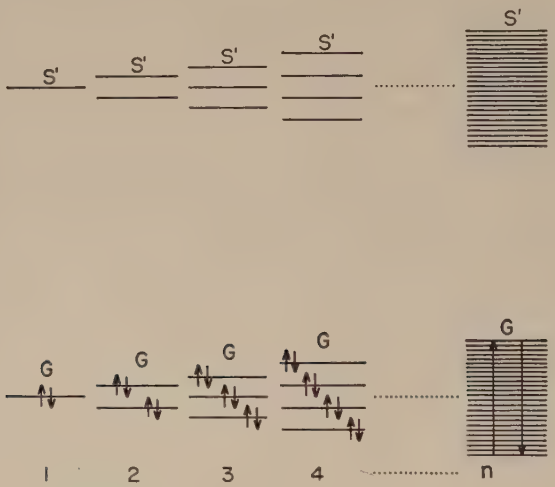

FIGURE 2. Schematic representation of the formation of energy bands in crystals through the interaction of n molecules. The pairs of arrows represent electrons with antiparallel spins. G = ground state; S′ = lowest excited singlet state.

Birks.[24] There is some disagreement as to the importance of this mechanism,[29, 30] and it has been shown in a number of instances to be of little significance.[31, 32]

(2) Resonance transfer analogous to unordered systems.[33, 34] According to this theory, the energy is transferred by an overlap of the electronic systems of the excited donor and the unexcited acceptor molecules. In this case, the interaction between molecules is sufficiently small to permit them to be considered as individual electronic systems. The probability of transfer increases with the magnitude of the interaction.

(3) The migration of excitons throughout the crystal.[35-41] The main features of this theory may be understood by a consideration of FIGURE 2. The interactions between the π orbitals of the n molecules in the crystal are so large as to lead to a splitting of the energy levels, resulting in the formation of n closely spaced levels. In general, the lower bands of levels are completely occupied by electrons, whereas the upper bands are vacant. Absorp-

tion of a quantum of light raises an electron from the lowest, or ground-state, band into the upper, or singlet-state, band. Inasmuch as the levels in this upper band are very closely spaced, the thermal energy at most temperatures is sufficient to allow the electron to move from any one level in the band to any other level. Thus, the excited state cannot be considered as belonging to any one molecule in the crystal, but rather must be considered as belonging to the crystal as a whole. We thus arrive at a concept of an excited state free to migrate throughout an ordered array of molecules. Such an excited state can be visualized as consisting of a negatively charged electron in the upper, or excited, band and a positively charged hole—the vacancy left by the electron when it is raised into the upper band—in the lower, or

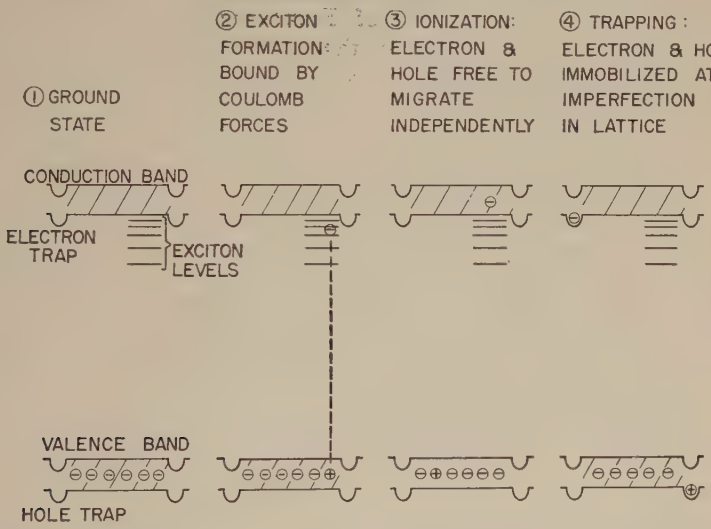

FIGURE 3. Schematic representation of conduction bands and trapping levels in an ordered array of molecules.

valence, band. This is shown schematically in FIGURE 3. The components of the electron-hole pair attract each other through ordinary Coulomb forces and migrate as a unit throughout the crystal. Such a state of the crystal is called an exciton, inasmuch as it is formally equivalent to a neutral, massless particle with spin zero traveling through the crystal. It is apparent that it is possible to have triplet excitons as well as singlet excitons, although normally the triplet state will be split to a far lesser extent than the corresponding singlet state.

In an ideal crystal, such an exciton would migrate throughout the crystal until either it recombined, with the emission of a quantum of radiation, or its energy became degraded into the lattice vibrations of the crystal. However, all real crystals contain imperfections in the lattice structure resulting from such factors as dislocations, vacancies, and impurities. Imperfections of this type cause some of the excitons to be ionized, that is, the electron and the

hole are no longer constrained to migrate as a unit, but each is capable of moving independently of the other. Furthermore, the crystal imperfections give rise to trapping centers that are capable of immobilizing the electrons and/or holes. These traps may be considered as energy levels lying somewhat below the lowest level of the conduction band. Ultimately, the electrons and holes recombine with each other, but it is quite possible for them to have very different histories before recombination, spending various amounts of time trapped in impurities and imperfections in different parts of the crystal. In general, the most mobile entities in organic crystals are the holes.[42]

There is some controversy in the literature on whether exciton migration or resonance transfer is the most significant mechanism for energy transfer in ordered systems.[24] The objections of Franck and Livingston[33] and of Livingston[34] to the exciton theory in the case of anthracene crystals are primarily that in its terms one cannot account for the absorption and emission spectra of the pure crystals. However, it must be pointed out that an adequate quantum-mechanical interpretation of the absorption spectrum of crystalline anthracene has been given by Davydov[38] and by Craig and Hobbins[43] on the basis of exciton theory. Furthermore, recent work on the photoconductivity (see below) of anthracene crystals and on the sensitized fluorescence of impurities in anthracene crystals[44] suggests that both these phenomena represent alternative pathways for the degradation of an exciton, which probably takes place at a dislocation in the crystal.

Experimental aspects. The above discussion, while greatly oversimplified, enables one to achieve a good deal of insight into many of the electronic properties of organic crystals. These include:

(1) Photoconductivity: the electrons and holes formed as a result of the absorption of light, being free to migrate throughout the crystal, endow the crystal with the property of conducting an electric current. The exciton itself, being neutral, does not contribute to the photoconductivity.

(2) Semiconductivity: at room temperature, the thermal energy is normally insufficient to achieve a very large dark population of the conduction band in most crystals. However, as the temperature is raised, more and more electrons are excited into the conduction band in accordance with Boltzmann's law. Thus, many crystals that are insulators at low temperatures exhibit an increasing conductivity as a function of temperature.

(3) Luminescence: there are, in general, four main mechanisms of luminescence in organic crystalline semiconductors; not all of these need be operative simultaneously. They are: (*a*) direct decay of the exciton (fluorescence or phosphorescence); (*b*) recombination and radiative decay of the electron and hole subsequent to ionization, but prior to trapping; (*c*) excitation of the trapped electron and/or hole into the conduction band, followed by recombination and radiative decay; and (*d*) transfer of the excitation energy to a fluorescent impurity in the crystalline lattice (sensitized fluorescence).

Luminescence processes *a*, *b*, and *c* all lead to emissions of the same wave length, but with different time constants and temperature dependencies. Process *a* is relatively temperature independent; process *b* may or may not

exhibit a temperature coefficient, depending on the actual mechanism of ionization; process *c* has a very definite temperature dependence as a function of the depth of the traps.

(4) Thermoluminescence: if the trap depths are such that, at a given temperature, the excitation of the trapped electron or hole into the conduction band does not proceed at a measurable rate, irradiation followed by an increase in temperature leads to luminescence. Under such conditions, the luminescence-versus-temperature curve of the crystal exhibits peaks corresponding to the various trap depths.

A typical example of an energy-transfer process in molecular crystals is given by the study on anthracene crystals by Bowen *et al.*[45] They found that the presence of 0.1 per cent of naphthacene in anthracene almost completely quenches the blue-violet fluorescence of anthracene and replaces it with the yellow-green emission of naphthacene. The quantum yield of this process is only slightly less than that of the fluorescence of pure anthracene. Similarly, traces of anthracene in naphthalene replace the U.V. fluorescence of the naphthalene with the blue-violet anthracene fluorescence. The semiconductivity and photoconductivity of anthracene have been quite extensively studied,[44] and—of somewhat more biological interest—similar studies have been carried out on the phthalocyanines.[46]

A very interesting series of experiments by Borthwick *et al.*,[47] Evanari and Stein,[48] and others[49, 50] on the germination of seed has shown that red light (5250 Å to 7000 Å) stimulates such germination, whereas infrared radiation (7000 Å to 8200 Å) reverses this effect. These effects show a marked resemblance to phenomena exhibited by many crystalline phosphors; in fact, the suggestion has been made that these phenomena are the result of the formation of trapped electrons in a semiconducting system by the action of the red light and of the detrapping of the electrons by the infrared light. It will be interesting to see if further experimentation supports this viewpoint.

Energy Transfer in Green Plant Materials

Katz,[51] in 1949,* and Bradley and Calvin,[53] independently, in 1955, suggested that aggregates of chlorophyll molecules in the chloroplasts might give rise to conduction bands in which photoproduced electrons and holes could migrate. Such a system would have the advantage of providing for a separation of the oxidizing and reducing entities known to be necessary for photosynthesis.

This concept has remained purely speculative until the quite recent publication of a number of researches suggesting that something of this nature may indeed take place within chloroplasts. In 1956, Commoner *et al.*[54] published evidence for the presence of a light-induced electron spin resonance (ESR) in spinach chloroplasts due to the photoproduction of unpaired electrons. Again, in 1957, these workers have shown the presence of two kinds of unpaired spins, one of which is transformed into the other.[55]

* In an earlier publication,[52] Szent-Györgi wrote of "conduction bands" in proteins as possibly significant in photosynthesis, but this concept does not correspond to the pi-electron system we are discussing here.

In 1957, Arnold and Sherwood[56] studied dried chloroplast films and found that they exhibited semiconductivity and thermoluminescence. In addition, some studies by Strehler and others[57-60] have demonstrated the existence of temperature-dependent long-lived luminescences in algae and in chloroplasts. Finally, the photoconductivity of chlorophyll films has been observed.[61]

Our own experiments in this area began in 1956 with the demonstration by Sogo *et al.*[62] of a light-induced ESR signal in dried eucalyptus leaves. Inasmuch as these results were rather poorly reproducible, it was decided to study isolated chloroplasts. Furthermore, when it became apparent that the spin-resonance signals decayed fairly rapidly when the light was turned off, the possibility that at least part of the energy associated with these unpaired

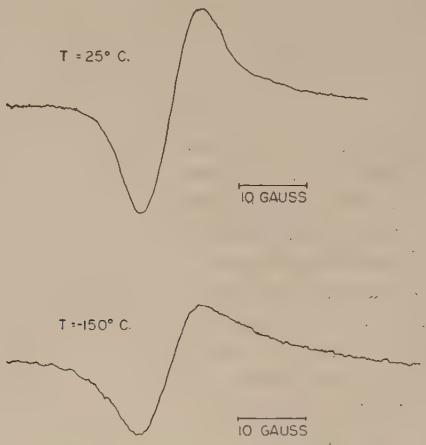

FIGURE 4. Light signals from whole spinach chloroplasts.

spins might appear as luminescence led us to a study of the light-emission properties of the chloroplasts.[63]

The chloroplasts are prepared by grinding spinach leaves in a blender and carrying out a series of differential centrifugations.[62] These enable us to obtain what we shall call intact chloroplasts and large and small chloroplast fragments.

Two typical ESR curves for wet whole spinach chloroplasts are shown in FIGURE 4. These curves are essentially derivative plots of microwave power absorbed in the sample versus the magnetic field strength. These signals represent approximately 10^{16} unpaired spins. The wave lengths of light effective in exciting these signals are between 3500 Å and 4500 Å and between 6000 Å and 7000 Å, indicating absorption by chlorophyll. A rough quantum-yield measurement indicates a value lying between 0.1 and 1.

FIGURE 5 shows some results of growth- and decay-time measurements on the samples. In this case, the curves represent power absorption versus time at constant magnetic field strength. An analysis of the 25° decay curve (FIGURE 6) produced the two decay times of 1 sec. and 10 sec. The

room-temperature rise time for the light-produced signal is less than the 0.2-sec. response time of the detection apparatus. At −150° C., essentially the same rise time is observed, but the decay time is of the order of hours. This effect of cooling is completely reversible. With dried chloroplasts at

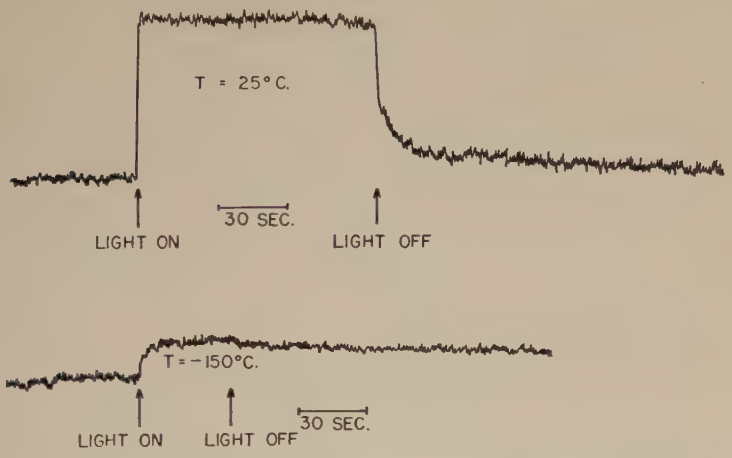

FIGURE 5. Growth and decay curves of whole spinach chloroplasts at T. = 25° C.

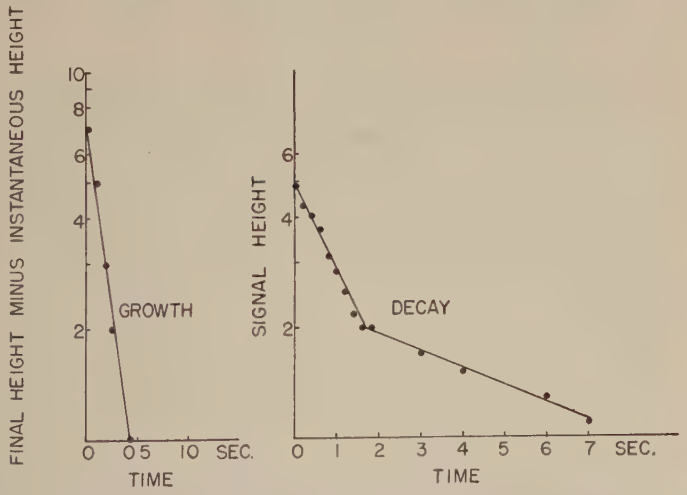

FIGURE 6. Analysis of growth and decay curves of whole spinach chloroplasts at T. = 25° C.

25° C., the rise times are similar, but the decay times are of the order of hours. However, at 60° C. the decay time of the dried material is of the order of seconds. These figures are summarized in TABLE 1.

Some of the luminescence decay curves for wet whole spinach chloroplasts are shown in FIGURE 7. The apparatus is so designed that we are able to

TABLE 1
COMPARISON OF ESR AND LUMINESCENCE OBSERVATIONS ON CHLOROPLASTS

Material	Temp. (°C.)	Relative ESR light signal	Rise time		Decay time	
			ESR*	600–800 mμ luminescence*	ESR*	600–800 mμ luminescence*
Wet fresh chloroplasts	25	3	<0.2 sec.†	<0.1 sec.†	≤1 sec.† (60%) 10 sec. (40%)	0.15 sec. (6%) 2 sec. 15 sec. } (94%)
	−35	9	<1 sec.† (75%) 12 sec. (25%)	<0.1 sec.†	≤1 sec.† (33%) 10 sec. (33%) 3 min. (33%)	0.15 sec. (75%) 2 sec. (25%)
	−140	4	<1 sec.†	no signal	~ hr.	no signal
Dried chloroplasts	25	—	~ min.	no signal	~ hr.	no signal
	60	—	~ sec.	—	~ sec.	~ sec.

* Excited by wave lengths between 350 and 450 mμ or 600 and 700 mμ.
† Instrument limited.

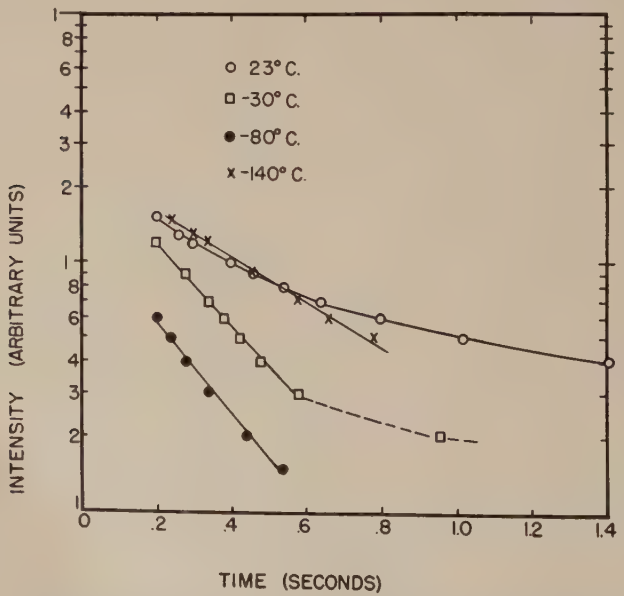

FIGURE 7. Luminescence decay curves for wet whole spinach chloroplasts at four temperatures. Log intensity is plotted against time.

observe continuously the light emitted from the chloroplasts approximately 0.1 sec. after excitation by a flash of light.[63] An analysis of these curves and those for intermediate temperatures demonstrates that the room-temperature emission consists of at least three components having different temperature dependencies and having half lives of 0.15, 2, and 15 sec., respectively. Approximately 6 per cent of the total integrated light intensity up to about 7 sec. after the flash is due to the 0.15-sec. emission. When the chloroplasts are cooled, the slower components diminish in intensity and vanish at about −40° C. At this temperature, the decay curve is the same as that obtained by subtracting the slower components from the room-temperature curve. When the chloroplasts are cooled still further, the 0.15-sec. component diminishes in intensity, its decay constant apparently remaining approximately the same, and is gone at about −100° C.

The excitation and emission spectra of the luminescence were measured using a Bausch and Lomb grating monochromator between the flash and the sample and between the sample and the detector (Tollin *et al.*, to be published). The curves are shown in FIGURE 8. The action spectra for *Chlorella*[57] and for spinach chloroplasts are quite similar to the absorption spectra of these materials. The action spectrum for *Nostoc*, on the other hand, shows a relatively low activity for chlorophyll and carotenoids and a high activity for phycocyanin. Thus, electronic excitation energy may be transferred, with some degree of efficiency, from carotenoid to chlorophyll in *Chlorella* and in spinach chloroplasts, but such transfer occurs only poorly in *Nostoc*.

The room-temperature emission spectra of thin films of *Chlorella*[64] and of chloroplasts demonstrates that the luminescence is the result of a transition between the first excited singlet state and the ground state of chlorophyll. The somewhat broadened shape of the luminescence spectra as compared to a typical fluorescence spectrum is the result of the relatively large monochromator slit widths necessitated by the low intensity of the luminescence. Filter experiments indicate that the 0.15-, 2-, and 15-sec. emissions all have the same spectral distribution.

In an earlier publication,[63] it was tentatively suggested, on the basis of measurements with filters on thick films of material, that the luminescence of spinach chloroplasts was the result of a triplet-state to ground-state emission of chlorophyll. This is now recognized as having been due largely to the self-absorption distortion of the emission spectrum.

Emission spectra for thick films of spinach chloroplasts at three temperatures are also plotted in FIGURE 8. Thick films were used in order to compensate for the low intensity of the low-temperature emissions (see above). The markedly different shape of these spectra, as compared with the thin-film spectra, is due to self-absorption. However, it is apparent that the curves at all three temperatures are identical in shape, which suggests that they are the result of the same electronic transition. This temperature independence of the spectrum of emission indicates that the triplet state of chlorophyll is not involved in the delayed light emission of spinach chloroplasts.

The luminescence of *Nostoc* is far weaker than that of either *Chlorella* or of

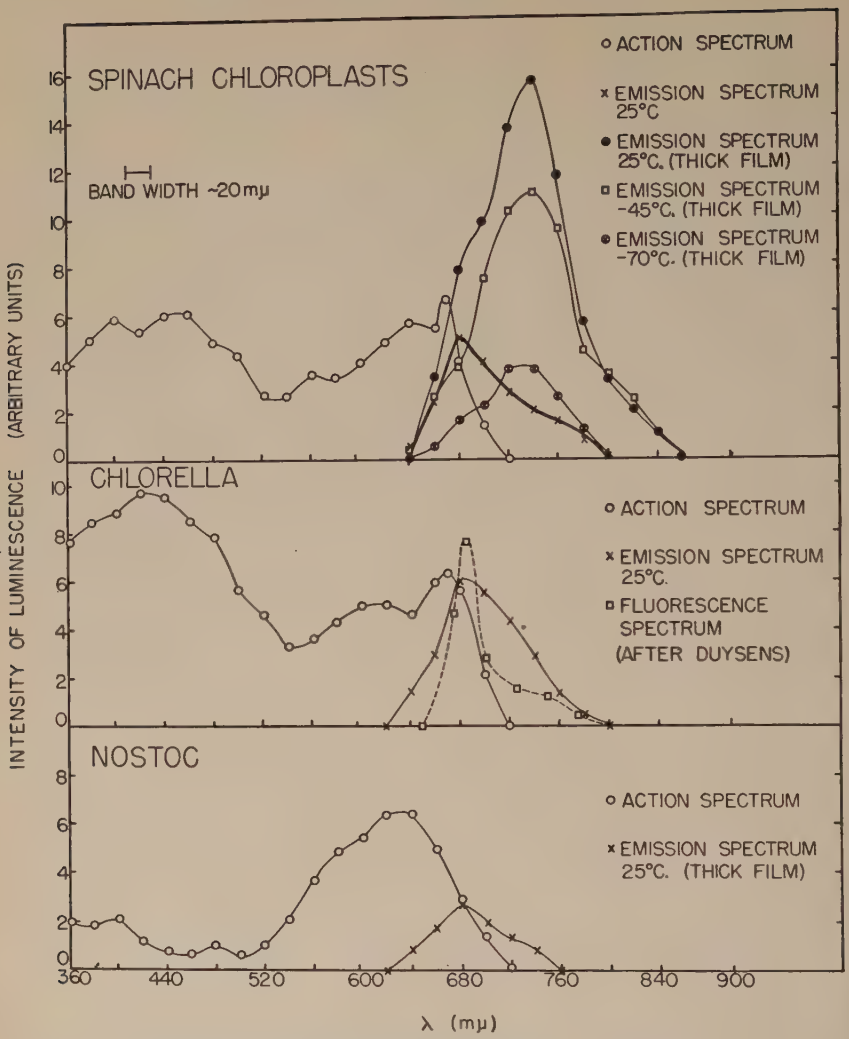

FIGURE 8. Excitation and emission spectra for spinach chloroplasts, *Chlorella*, and *Nostoc*.

spinach chloroplasts, necessitating the use of thick films of material. In view of this, one would expect that if the emission were due solely to chlorophyll, the spectrum would be similar to those obtained with thick films of chloroplasts. However, this is not actually the case, which suggests that a significant portion of the emitted light originates in phycocyanin, which has a fluorescence peak at about 660 mμ and is self-absorbed in the thick layer.

FIGURE 9 shows the effects of allowing freshly prepared chloroplasts to stand in the dark at 23° C. Up to 8 hours, the luminescence gradually increases in intensity, and reaches a maximum intensity 2.7 times that of

freshly prepared material. This larger signal exhibits the same decay curve, wave length properties, and temperature behavior as does the original signal. Allowing the chloroplasts to stand still longer decreases the luminescence intensity and causes changes in the decay curve. After about 72 hours the luminescence disappears entirely, and the chloroplasts exhibit thermoluminescence similar to that observed by Arnold and Sherwood for quick-dried chloroplasts.[56]

Although it is not possible at this time quantitatively to compare the ESR results with the luminescence results, there are a number of significant qualitative similarities, which are summarized in TABLE 1 (although it should

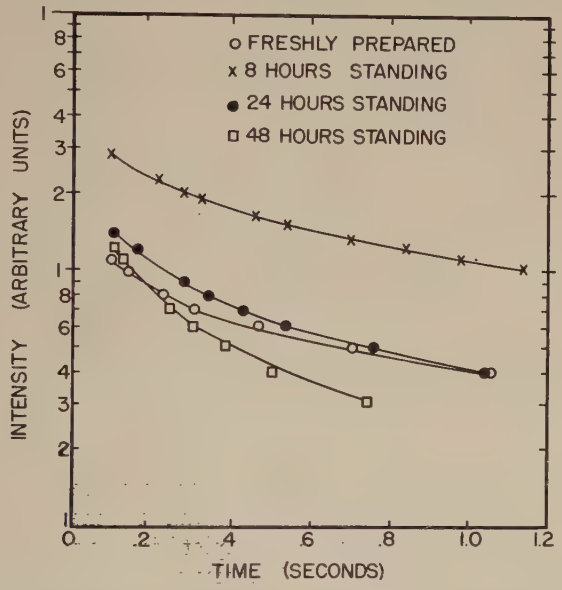

FIGURE 9. Effects of allowing freshly prepared wet whole spinach chloroplasts to stand in the dark at 23° C.

be kept in mind that the types of excitation in the two experiments are quite different):

(1) Both phenomena are excited by the same bands of wave lengths and both are due to absorption by chlorophyll.

(2) The 25° C. decay times for wet chloroplasts are of the same order of magnitude for both phenomena.

(3) At −140° C. the ESR decay times are of the order of hours, and no luminescence could be detected (a luminescence with a decay time of the order of hours would be undetectable with the apparatus used in the studies reported here).

(4) At 25° C. the decay time of the ESR for dried chloroplasts is of the order of hours, and under similar conditions the chloroplasts did not luminesce.

(5) At 60° C. the ESR of the dried chloroplasts had a decay time of the

order of seconds. At this same temperature, we have observed a peak in the thermoluminescence of the dried chloroplasts.

The above similarities strongly suggest that the 6000 to 8000 Å light emission of chloroplasts is at least in part the result of the decay of some of the unpaired spins detected by the ESR experiments. That some of the radicals decay by nonluminescent processes is indicated by the fact that the light emission at −35° is smaller than at room temperature. A quantitative comparison of the quantum yields, action spectra, and kinetic constant of these two phenomena is now being carried out. This should lead to a more definitive assessment of the relationships between them.

There are four possible mechanisms for the production of either ESR or delayed light emission in systems of the type we are concerned with here: (1) The production of radicals by the direct photodissociation of a single bond involving migration of the fragments, followed by their recombination in the dark; (2) the excitation and decay of a triplet state; (3) the reversible photosensitization of chemical or enzymatic processes leading to the production of free radicals; and (4) production of trapped electrons in a quasi-ordered lattice. Let us now see what can be said with regard to an interpretation of the present experimental results in terms of these mechanisms.

Mechanism 1 is inconsistent with the following considerations. No known stable and naturally occurring chemical bond can be dissociated by 6000 to 7000 Å light. Furthermore, decay times of the order of many seconds are not in the range to be expected for radical recombinations at relatively high temperatures. Finally, it is difficult to reconcile such a mechanism with the existence of three separate emissions of the same wave length.

The excitation and decay of a long-lived triplet state, as in Mechanism 2, is inconsistent with the observed definite temperature dependence of the chloroplast luminescence, that is, it is very unlikely that lowering the temperature to −140° C. would increase the triplet lifetime to the order of hours, as required both by the spin resonance studies and by the fact that no triplet emission can be observed. Furthermore, such a mechanism cannot result in three separate emission acts having different time constants, but of the same wave length.

Cooling to −140° C. should decrease the rates of any chemical or enzymatic processes occurring here, as in Mechanism 3, to essentially zero. Thus, the unpaired spins produced at this temperature cannot be considered as having arisen in this manner. On the other hand, the ESR results at −35° C. (that is, the appearance of a 12-sec. rise time) suggest that part of the radicals formed at that temperature and at room temperature are due to chemical transformations. The larger spin signal at −40° C. may be accounted for by assuming a greater temperature coefficient for the decay of these chemically produced radicals than for their formation.

Thus Mechanism 4 seems a likely explanation for the primary process of the phenomena we are reporting here. We shall next see how such a scheme fits the data.

FIGURE 10 is a schematic representation of the electronic energy bands in

chloroplasts. Inasmuch as the band width will be proportional to the square of the transition probability for the ground-state to excited-state transition,[65] the excited singlet state will be much broader than the corresponding triplet state. As a result, there may be a good deal of overlap between the energy levels of these two states. It is necessary to postulate such overlap in order to provide a relatively temperature-independent pathway between the states to account for the inability to observe triplet-state emission, even at $-70°$ C.

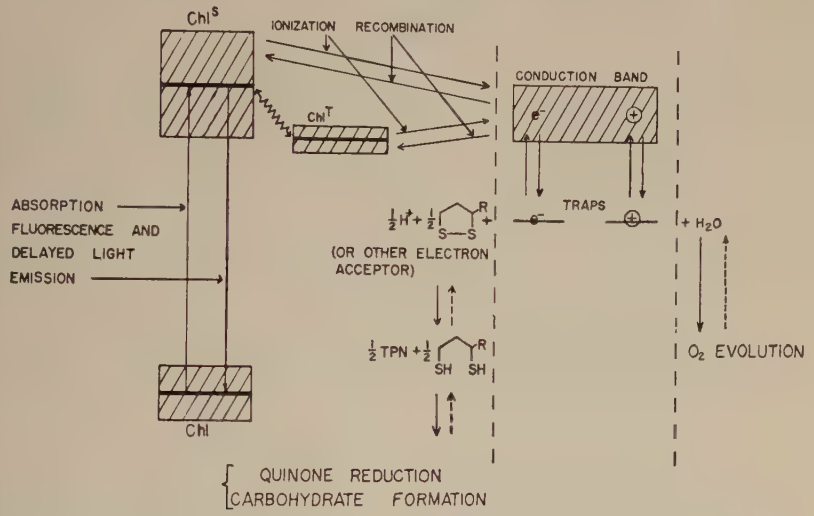

FIGURE 10. Proposed scheme for various photochemical processes in photosynthesis.

Light is absorbed to produce the transition from the ground-state band of an aggregate of chlorophyll molecules to the first, excited singlet-state band. Singlet-state excitons may then undergo one of three competing processes:

(1) They may decay to the ground state via fluorescence emission ($\tau \cong 10^{-9}$ sec.).

(2) They may ionize with the formation of electrons and holes in conduction bands ($\tau < 10^{-9}$ sec.). Calculations have shown that such a lifetime would permit the exciton to migrate over from 100 to 1000 molecules.[28]

(3) They may cross over in a radiationless transition into the triplet state in times as short as 10^{-12} sec.

If the triplet-state conversion is important in chloroplasts, ionization into the conduction bands may occur from this state. The electrons and holes in the conduction band will migrate and ultimately be trapped at suitable points in the lattice. Characteristic lifetimes of 0.01 to 0.1 sec. have been observed in many types of experiments on photosynthetic materials.[53, 59, 62, 63] According to the present hypothesis, this would represent the time required for the population of the traps. If ionization occurs from the singlet state, this time constant may be identified with the lifetime of one of the charge carriers in the conduction band. Such a hypothesis has some support from

the fact that, for germanium, the intrinsic minority carrier lifetime is calculated to be 0.75 sec.[66] Experimentally, lifetimes on inorganic semiconductors may range from 10^{-13} sec. to several seconds.[67] No corresponding measurements have been made for organic semiconductors. If, on the other hand, ionization occurs from the triplet state, the 0.01- to 0.1-sec. time constant may represent either the ionization time constant or a carrier lifetime.

It is not possible, at present, to decide which of the two mechanisms, direct ionization from the singlet state or ionization from the triplet state, is operative in chloroplasts. Indeed, it may be that both processes occur simultaneously.

The number of traps in the chloroplast is probably very small, perhaps of the order of one per several thousand chlorophyll molecules. Thus, this scheme leads directly to the idea of a "photosynthetic unit."[68] The electrons and holes that are trapped give rise to a spin-resonance signal. The traps are thermally depopulated, the resultant electrons and holes in the conduction band recombine, and a temperature-dependent luminescence results. Such recombination may occur directly into the singlet state or into the singlet state via the triplet state. The 2- and 15-sec. lifetime emission may be identified with the depopulation of traps of different depths. The 0.15-sec. decay may represent either the depopulation of a shallow trap or the lifetime of one of the charge carriers in the conduction band. Further experimentation is in progress to determine the nature of the 0.15-sec. decay, as well as the 0.01-sec. decay reported by Arthur and Strehler.[59]

At low temperature, the thermal energy is insufficient to excite the electrons and holes out of the traps, and enzymatic production and decay of radicals no longer occurs. This results in the disappearance of the luminescence and the appearance of a long-lived ESR signal. The thermoluminescence referred to earlier may be the result of a deepening of the trapping levels due to drying.

The electrons and holes in the traps may also be used up by enzymatic processes. Any reversibility in these enzymatic processes would then lead to a long-lived luminescence, which could be classified as a chemiluminescence. It is likely that some of the longer-lived emissions reported by Strehler and Arnold[57] and Strehler[58] are of this nature, and perhaps also the 15-sec. emission reported here. If these enzymatic processes involve free radicals, similar decay times will occur in the spin-resonance analysis. The fact that almost three times more light energy is emitted in aged chloroplasts as in fresh chloroplasts suggests that these enzymes are easily inactivated. A similar increase in the number of light-induced radicals in aged chloroplasts is found in spin-resonance experiments. These observations suggest that enzymatic utilization represents the normal pathway for most of the electrons and holes in the living cell. In this way the light energy could be made available to the photosynthetic mechanism.

References

1a. Franck, J. & F. Cario. 1922. Z. Physik. **11**: 161.
1b. Franck, J. & F. Cario. 1923. Z. Physik. **17**: 202.

2. Franck, J. & E. Teller. 1938. J. Chem. Phys. **6**: 861.
3. Vavilov, S. I. 1925. Z. Physik. **31**: 750.
4. Pringsheim, P. 1949. Fluorescence and Phosphorescence. : 60–63. Interscience. New York, N. Y.
5. Kallman, H. P., M. Furst & F. H. Brown. 1956. Symposium on fluorescence and semiconductors, Garmisch-Partenkirchen, Germany.
6. Furst, M. & H. P. Kallman. 1954. Phys. Rev. **96**: 902.
7. Pringsheim, P. 1954. Phys. Rev. **96**: 99–100.
8. Kasha, M. 1952. J. Chem. Phys. **20**: 71.
9. Kasha, M. & S. P. McGlynn. 1956. Ann. Rev. Chem. **7**: 403.
10. Hardwick, R. 1957. J. Chem. Phys. **26**: 323.
11. Kallman, K. & F. London. 1928. Z. Physik. Chem. Leipzig. B(2) **207.**
12. Förster, T. 1948. Ann. Physik. **2**: 55.
13. Vavilov, S. 1943. J. Phys. USSR. **7**: 141.
14. Förster, T. 1951. Fluorescence organischer Verbindungen. Vanderhoeck & Ruprecht. Göttingen, Germany.
15. Rabinowitch, E. & L. Epstein. 1941. J. Am. Chem. Soc. **63**: 69.
16. Förster, T. 1949. Z. Elektrochem. **53**: 93.
17. Lavorel, J. 1957. J. Phys. Chem. **61**: 864.
18. Watson, W. & R. Livingston. 1950. J. Chem. Phys. **18**: 802.
19. Duysens, L. 1951. Nature. **168**: 548.
20. Shore, V. G. & A. B. Pardee. 1956. Arch. Biochem. Biophys. **62**: 355.
21. Terenin, A. & V. Ermolaev. 1952. Doklady Akad. Nauk SSSR. **85**: 547.
22. Ermolaev, V. 1955. Doklady Akad. Nauk SSSR. **102**: 925.
23. Terenin, A. & V. Ermolaev. 1956. Trans. Faraday Soc. **52**: 1042.
24. Birks, J. B. 1953. Scintillation Counters. Pergamon. London, England.
25. Wassink, E. C. 1948. Enzymologia. **12**: 362.
26. French, C. S. & V. K. Young. 1952. J. Gen. Physiol. **35**: 873.
27. Arnold, W. & E. S. Meek. 1956. Arch. Biochem. Biophys. **60**: 82.
28. Rabinowitch, E. 1957. J. Phys. Chem. **61**: 870.
29. Bowen, E. J. & P. D. Lawley. 1949. Nature. **164**: 572.
30. Bowen, E. J. 1951. Symposia Soc. Exptl. Biol. **No. 5**: 152.
31. Ferguson, J. 1956. Australian J. Chem. **9**: 160.
32. Ferguson, J. 1956. Australian J. Chem. **9**: 172.
33. Franck, J. & R. Livingston. 1949. Revs. Modern Phys. **21**: 505
34. Livingston, R. 1957. J. Phys. Chem. **61**: 860.
35. Frenkel, J. 1931. Phys. Rev. **37**: 17.
36. Peierls, R. 1922. Ann. Physik. **13**: 905.
37. Franck, J. & E. Teller. 1938. J. Chem. Phys. **6**: 861.
38. Davydov, A. S. 1948. J. Exptl. Theoret. Phys. USSR. **18**: 210.
39. Curran, S. C. 1953. Luminescence and the Scintillation Counter. Chaps. 6 & 7. Academic Press. New York, N. Y.
40. Kittel, C. 1956. Introduction to Solid State Physics. Chaps. 13, 17 & 18. Wiley. New York, N. Y.
41. Leverenz, H. W. 1950. An Introduction to the Luminescence of Solids. Wiley. New York, N. Y.
42. Kallman, H. P. & M. Silver. 1956. Symposium on Fluorescence and Semiconductors, Garmisch-Partenkirchen, Germany.
43. Craig, D. P. & P. C. Hobbins. 1955. J. Chem. Soc. : 539.
44. Compton, D. M. J., W. G. Schneider & T. C. Waddington. 1957. J. Chem. Phys. **27**: 160.
45. Bowen, E. J., E. Mikiewicz & F. Smith. 1949. Proc. Phys. Soc. London. **62**: 26.
46. Fielding, P. E. & F. Gutman. 1957. J. Chem. Phys. **26**: 411.
47. Borthwick, H. A., S. B. Henricks, M. W. Parker, E. H. Toole & V. Toole. 1952. Proc. Natl. Acad. Sci. U. S. **38**: 662.
48. Evenari, M. & G. Stein. 1953. Experientia. **9**: 94.
49. Evenari, M., G. Neumann & G. Stein. 1953. Nature. **172**: 452.
50. Stein, G. 1954. J. chim. phys. **51**: 133.

51. Katz, E. 1949. In Photosynthesis in Plants. W. E. Loomis and J. Franck, Eds. Chap. 15. : 291. Iowa State College Press. Ames, Iowa.
52. Szent-Györgi, A. 1941. Science. **93**: 609.
53. Bradley, D. F. & M. Calvin. 1955. Proc. Natl. Scad. Sci. U. S. **41**: 563.
54. Commoner, B., J. J. Heise & J. Townsend. 1956. Proc. Natl. Acad. Sci. U. S. **42**: 710.
55. Commoner, B., J. J. Heise, B. B. Lippincott, R. E. Norberg, J. V. Passoneau & J. Townsend. 1957. Science. **126**: 57.
56. Arnold, W. & H. K. Sherwood. 1957. Proc. Natl. Acad. Sci. U. S. **43**: 105.
57. Strehler, B. L. & W. Arnold. 1951. J. Gen. Physiol. **34**: 809.
58. Strehler, B. L. 1951. Arch. Biochem. Biophys. **34**: 239.
59. Arthur, W. E. & B. L. Strehler. 1957. Arch. Biochem. Biophys. **70**: 507.
60. Strehler, B. L. & V. K. Lynch. 1957. Arch. Biochem. Biophys. **70**: 527.
61. Nelson, R. C. 1957. J. Chem. Phys. **27**: 864.
62. Sogo, P. B., N. G. Pon & M. Calvin. 1957. Proc. Natl. Acad. Sci. U. S. **43**: 387.
63. Tollin, G. & M. Calvin. 1957. Proc. Natl. Acad. Sci. U. S. **43**: 895.
64. Arnold, W. & J. B. Davidson. 1954. J. Gen. Physiol. **37**: 677.
65. Simpson, W. T. & D. L. Peterson. 1957. J. Chem. Phys. **26**: 588.
66. van Roosbroeck, A. & W. Schockley. 1954. Phys. Rev. **94**: 1558.
67. Rose, A. 1956. *In* Atlantic City Conference on Photoconductivity. : 112. Wiley. New York, N. Y.
68. Gaffron, H. & K. Wohl. 1936. Naturwiss. **24**: 81–103.

Part III. Physiology of Photoreception

ELECTRICAL ACTIVITY OF A "PRIMITIVE" PHOTORECEPTOR*

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Most electrophysiological studies on visual systems have been performed on photoreceptors that are far from simple. For example, analysis of the primary excitatory process of the vertebrate eye has been complicated by the activity of the neural network of the retina and by the highly organized ultrastructure of the receptor cells themselves. Even the lateral eye of *Limulus*, which at first appeared attractively simple, presents complexities of unit structure, as well as of interaction between units. This paper describes some experiments on the caudal photoreceptor of the crayfish, which fulfills at least some of the criteria for function simplicity. Perhaps some excuse should be offered for the use of the adjective "primitive" to describe the photoreceptor, which is the subject of these experiments. The word does not necessarily imply a primitive status in the evolution of visual systems; rather, it refers to the likelihood that the units studied represent only slightly modified neural elements.

The photoreceptor in question is located in the sixth abdominal ganglion of the crayfish ventral nerve cord. This ganglion lies immediately under the ventral exoskeleton, which is usually transparent; it is thus in a position favorable for receiving direct stimulation under natural conditions.

Discharge Pattern and Properties

Prosser (1934) was the first to note that illumination of the sixth ganglion produced a tonic increase in the spontaneous activity characteristic of the nerve cord. In my own experiments, cords were excised and stored in cold van Harreveld's solution to eliminate spontaneous activity. The sensory discharge was recorded with wick electrodes. In some cases, external potassium concentration was reduced, to diminish the spontaneous activity even further; but it was kept at a level at which the illumination thresholds and the discharge pattern were normal. Single afferent fibers were also isolated from the region between the sixth and fifth ganglia by fine dissection in intact preparations; such fibers showed spontaneous activity of a constant frequency, but their discharge pattern was identical in all other respects to that of units in the excised preparations.

The firing pattern of the 2 to 4 afferent fibers in the nerve cord that carry the photoreceptor discharge has already been described (Kennedy, 1958). Briefly, it exhibits the following features: (1) extremely long latency at the threshold for constant illumination—values of from 8 to 12 seconds are usual; (2) very slow adaptation during a continuous stimulus; (3) a "fast" frequency

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peak at high stimulus intensities, followed by a transient depression of activity and then a steady firing rate; and (4) pronounced afterdischarge, which often lasts for more than a minute after a brief stimulus of moderate intensity. Some of these properties are illustrated in FIGURE 1.

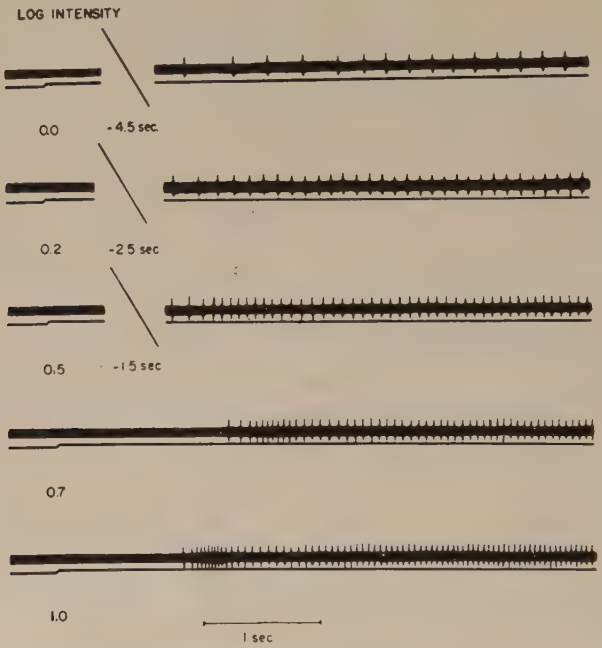

FIGURE 1. Discharge pattern of single afferent photoreceptor fibers in excised cord. The preparation was in potassium-free perfusion fluid; the record was made after the threshold had begun to rise, but before any changes in discharge pattern had occurred. The lower trace is the response of a monitoring photocell. (This and the following records were retouched for photographic purposes.)

Summation

The long latency and the prominent afterdischarge of these photoreceptor elements suggest that some phase of the excitation process has rather long time constants, both of development and of decay. Some information about the nature of these can be obtained from experiments employing paired flashes near threshold, with varying intervals between them. Paired flashes having equal, barely subthreshold intensity will summate to produce discharge over rather long time intervals (FIGURE 2). In most preparations, stimulus intervals of less than 1 second produce a large summated discharge containing up to 70 impulses. As the interval between flashes is increased, the number of impulses discharged in response to the second flash decreases. The maximum summation interval—that at which the summated response consists of a single impulse—is between 10 and 12 seconds.

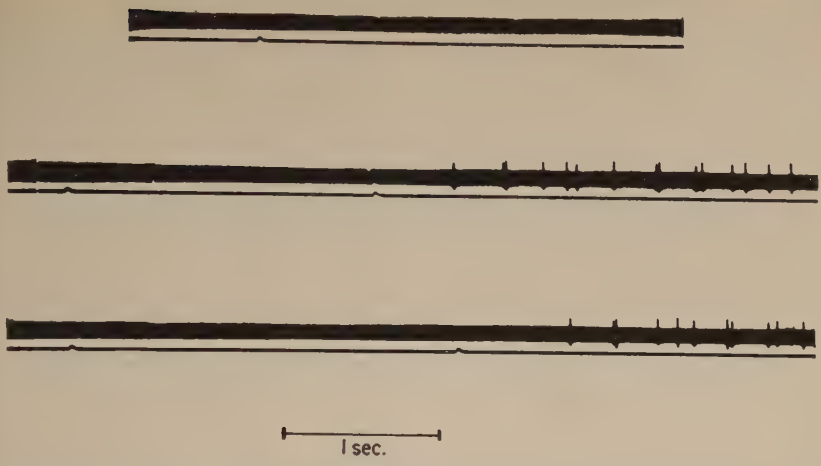

FIGURE 2. Summation of subliminal flashes. The flash duration was 0.02 sec.; intensity was adjusted to a value at which one flash just failed to elicit an impulse (upper record). Two different intervals are shown; the response latency increases and the frequency of discharge decreases as the interval becomes longer.

The intensity of both flashes together can also be adjusted to any desired level *above* threshold, in order to determine whether the occurrence of a response to the first flash removes its excitatory effects. In such an experiment, the first flash alone will generate a certain number of impulses; this will also represent, of course, the approximate number that the second flash would generate in the absence of any temporal summation. The results indicate clearly that such summation does occur. TABLE 1 summarizes a single experiment for a flash interval of 5 seconds.

The results of these two types of experiments can, with some difficulty, be compared quantitatively. This preparation is subjected to a series of flashes of increasing intensity to obtain a plot of log I versus number (or frequency) of impulses. The difference between the actual flash intensity and the intensity that would normally be required to produce a response of the frequency observed is an approximation of the change in threshold. A similar technique has been used by Hartline and McDonald (1947) in measuring threshold changes during dark adaptation.

TABLE 1
SUMMATION AT FIVE-SECOND FLASH INTERVAL

Log flash intensity	No. of impulses following 1st flash	No. of impulses following 2nd flash
0.1	0	0
0.2	0	11
0.3	0	33
0.4	9	70

The calculation of threshold change following subliminal stimuli requires the assumption that the threshold remains constant during any experiment. Although this is not usually achieved, the most recent experiments indicate that at intervals of less than 1 second the threshold for the second flash is decreased by at least 0.3 $\log_{10}$ units; this then declines to very small values at 10 to 12 seconds. It would be of interest to compare the alteration of second-flash thresholds in such cases with those in which the first flash is itself above threshold. There is a very narrow intensity range in which this can be done, even with the longest intervals because, with a small increase of intensity, the discharge generated by the first flash becomes longer than the stimulus interval. The lowering of threshold for the second

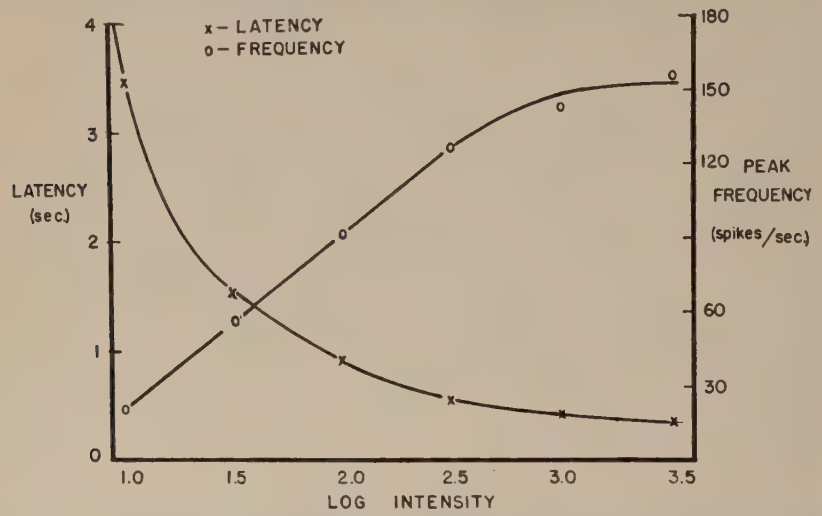

FIGURE 3. Relationship of latency and frequency to log intensity in a single experiment. The excised preparation was in van Harreveld's solution, and the stimulus was continuous.

flash appears to remain nearly constant as the number of impulses in the first-flash response is increased from 0 to about 20. This statement can be taken only as a qualitative estimate, however, since the threshold is not very stable and there is a constant background error due to sensitivity changes.

In any series of single-flash presentations, latency decreases and frequency increases as stimulus intensity is raised (FIGURE 3). The form of these variations is obviously quite different. Moreover, it has also been shown that, in a range of stimulus durations below 1.5 seconds, reciprocal flashes ($1 \times D = \text{const.}$) produce equal frequencies but markedly unequal latencies in such a manner that the latency to the brighter, shorter-duration flash is shorter. Similar differences in critical duration of frequency and latency have also been noted by Hartline (1934) for the *Limulus* ommatidium. Nevertheless, for a series of intensities at any given stimulus duration, there is a specific value of firing frequency for every value of latency; they are dependent variables. It can now be asked whether the summated responses

from paired-flash experiments show typical frequency-latency relationships for the duration employed. In TABLE 2, a series of single-flash and paired-flash experiments are compared. It can be seen that the latency values for the summated responses are far lower than the values for a single-flash response having the same discharge frequency.

TABLE 2

Log flash intensity	Frequency (impulses per second)	Number of impulses	Latency (seconds)
(A) Single flashes			
0.3	2	7	2.25
0.4	6	27	1.50
0.5	13	70	1.05
0.6	21	116	0.80
(B) Paired flashes: summated responses at three-second intervals			
0.1	—	1	2.75
0.2	4	16	0.85
0.3	10	43	0.55

These results may serve as the basis for some tentative speculations about the specific processes in the caudal photoreceptor that determine latency and frequency of discharge. One might assume that by some unspecified means the activation of photosensitive molecules within the receptor cell produces a depolarization that will bring about impulse discharge if it reaches a critical value. Such depolarizations have been observed in the *Limulus* ommatidium by Hartline *et al.* (1952). The slope of the depolarization will depend on intensity, and its duration, within limits, on the duration of the stimulus. Such a scheme is consistent with the differences in behavior of firing frequency and latency as intensity increases, although it does not account for the fact that frequency is linear with log intensity, rather than intensity. It is consistent with the finding that the latency of summated responses is short, since they originate from a "plateau" of excitation that may be very near the firing level.

Such a hypothesis implies that the tonic properties of this photoreceptor are related to the characteristics of the receptor membrane. One might account for them equally well by proposing a photochemical system with extremely slow kinetics, but two kinds of evidence argue against this interpretation. First, with respect to the sensitivity changes during light and dark adaptation, the rate of dark adaptation differs very little from that found in, for example, the *Limulus* ommatidium (Hartline and McDonald, 1947). Second, some attempts have been made to alter the excitable properties of the receptor membrane without affecting the photochemical system. When the concentration of potassium in the bathing fluid is reduced

to zero and several hours are allowed for the establishment of equilibrium through the sheath, marked changes occur in discharge pattern. The first effect is an increase in threshold; subsequently, adaptation rate increases, afterdischarge disappears almost completely, and the impulses tend to come

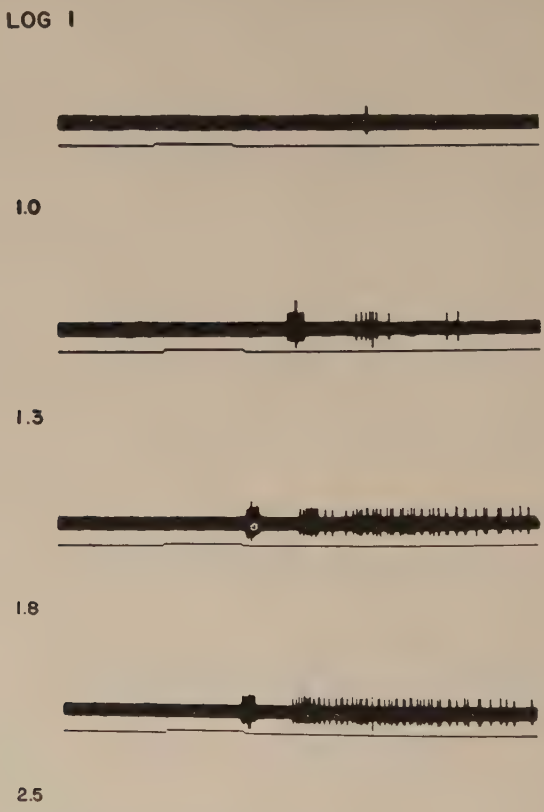

FIGURE 4. Photoreceptor discharge after several hours in potassium-free perfusion fluid. The stimulus duration was one second.

in "bursts" (FIGURE 4). Summation becomes restricted under these conditions. The results suggest, then, that neural properties, rather than photochemical ones, endow the caudal photoreceptor with its sluggish temporal characteristics.

Nature of the Receptor Elements

A comparison of the properties of the caudal photoreceptor with those of the *Limulus* ommatidium reveals that the former possesses far more tonic properties. These differences include, for example, the fact that maximum summation times are about 10 times as long, and that maximum and minimum latencies are about 10 times greater. Absolute threshold is several orders of magnitude higher in the caudal photoreceptor. In many of its

properties, this sense organ resembles slowly adapting mechanoreceptors more closely than it does other photoreceptors. It has been shown that afferent activation by the caudal photoreceptor is restricted to the small, spontaneously active interneurons of the crayfish, and never affects the "giant-fiber" system. These properties of discharge and of central activation suggest that the function of this receptor system is to supply information about the value of stimulus intensity over long periods of time.

This is exactly what one would expect a "primitive" photoreceptor to do and it is of interest to determine whether the sensory elements themselves demonstrate the lack of structural complexity that their properties suggest.

In excised cords that have been chilled to remove the spontaneous activity of other neural elements, the number of active afferent fibers rarely exceeds two; however, there is considerable variability among preparations in both absolute threshold and discharge pattern. Exploration with a small spot of light reveals that the sensory elements are restricted to the actual ganglion, but optical scattering prevents any more accurate localization by this technique.

In about one in four animals, a small spot of red pigment can be seen within the ganglion. Such pigment deposits, with one exception, have never been found in other ganglia of the ventral nerve cord. Microspectrophotometric measurements of this pigment reveal a maximum absorption of about 500 mμ; the curve agrees rather well with measurements of the spectral sensitivity of the photoreceptor units. While this agreement suggests the implication of the pigment in the photoreceptor processes, the weight of evidence is against such a view. First, the photoreceptor is obviously functional in pigmentless ganglia. Second, the pigment—although its absorption spectrum resembles that of rhodopsin—cannot be bleached, either by illumination or by wide pH alterations. It is probably not a rhodopsin at all, but astaxanthin, a carotenoid commonly found in decapod crustacea. Whether it plays any role at all in the activity of the photoreceptor is questionable.

Ungewitter's modification of the Bodian silver-impregnation technique (1951) has been employed recently in a histological search for specialized elements within the sixth ganglion. This technique can be applied to arthropod material with considerable success, and it allows a more adequate analysis than do the vital staining methods previously employed. Most of the dorsal region of the sixth ganglion is occupied by neuropil, a dense synaptic network of processes from entering sensory roots, ganglion cells, and cord elements. There are surprisingly few ganglion cells—less than 300—and they occupy the ventral region, where some of them form nuclei near the exit region of motor roots. These are, in general, large cells, ranging in soma diameter from perhaps 40 μ to more than 100 μ. They are all rather similar in appearance, and none with any distinguishably specialized characteristics have been seen. Since the technique is certainly capable of showing up whatever major differences in structure there may be, it seems reasonable to conclude that the photoreceptor structures may be slightly modified neurons equipped with a photosensitive pigment.

Summary

The discharge pattern of afferent photoreceptor fibers from the sixth abdominal ganglion of crayfish has been studied by recording electrically from excised nerve cords, or from single fibers in intact preparations. The receptor units show very long latencies at threshold, low adaptation rates, and pronounced afterdischarge.

Summation of brief sublimal flashes takes place over intervals as long as from 10 to 12 seconds, and rather long summated discharges (up to 70 impulses) can be produced when the interval between paired flashes is 1 second or less. The calculated threshold reduction for the second flash is at least 0.3 $\log_{10}$ units at this interval. Approximately similar threshold changes follow flashes that actually do generate impulses. It is also shown that the latency of the summated discharges is extraordinarily short.

Evidence is presented that suggests that the "tonic" properties of these receptor units depend on some membrane characteristic, rather than on a unique photochemical system.

The exact location and nature of the photoreceptor elements within the ganglion has not been determined. A photochemical system much like that of other visual organs appear to be present, and histological evidence suggests that the photoreceptor cells are actually slightly modified neural elements.

References

Hartline, H. K. 1934. Intensity and duration in the excitation of single photoreceptor units. J. Cellular Comp. Physiol. **5**: 229–247.

Hartline, H. K. & P. R. McDonald. 1947. Light and dark adaptation of single photoreceptor elements in the eye of *Limulus*. J. Cellular Comp. Physiol. **30**: 225–254.

Hartline, H. K., H. G. Wagner & E. F. MacNichol, Jr. 1952. The peripheral origin of nervous activity in the visual system. Cold Spring Harbor Symposia Quant. Biol. **17**: 125–141.

Kennedy, D. 1958. Response pattern and summation in a crustacean photoreceptor. (Abstr.) J. Cellular Comp. Physiol. In press.

Prosser, C. L. 1934. Action potentials in the nervous system of the crayfish. II. Responses to illumination of the eye and caudal ganglion. J. Cellular Comp. Physiol. **4**: 363–377.

Ungewitter, L. H. 1951. A urea silver nitrate method for nerve fibers and nerve endings. Stain Technol. **26**: 73–76.

STUDIES ON VISUAL CELL VIABILITY AND DIFFERENTIATION*

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Introduction

Several years ago two metabolic poisons, iodoacetate (IAA) and azide, were found to exert upon the retina of mammals effects of outstanding selectivity for certain cells of that tissue.[1-4] The two poisons had different effects, but each acted in so specific a manner that it seemed to reveal metabolic properties specifically related to the function of retinal elements. IAA, intravenously administered, affected electroretinogram and retinal excitation within less than twenty seconds after the injection, and abolished retinal function in response to illumination within a few minutes. If administered in doses higher than necessary for the block of retinal excitation, recovery was incomplete or nonexistent, and a fraction of the visual cell population or the whole population showed the signs of cell death after a few hours. These cells disintegrated and disappeared during the following days, but the other retinal cells—pigment epithelium, bipolar, and ganglion—survived. Azide, on the other hand, exerted its most selective effect on the steady (DC−) potential across the eye and on certain slow waves of the electrical response to illumination. It affected these potentials after injection the instant it reached the intraocular blood. The analysis of this effect led to the conclusion that azide probably acts upon the pigment epithelium by interfering specifically with an active ion transport system of this structure.[3, 4]

In the following, studies will be revised that originated from observation of the IAA effect. These studies were concerned with the organization of visual cell metabolism in regard to the maintenance of cell life and to sensory function. They involved the search for other poisons that, like azide or IAA, might help in defining the essential properties of retinal function and included attempts to analyze metabolic and functional changes that occur simultaneously with the anatomical differentiation of the visual cell. In agreement with recent findings on the microstructure[5-8] and histochemistry[9-12] of the visual cell, our studies lead to the conclusion that the spatial separation of this cell into different parts includes also a striking division in metabolic properties. This compartmentation of the visual cell into regions of different metabolic activities seems to impose special limitations on the viability of the cell. However, many phenomena of our studies that reveal peculiarities in functional and metabolic organization are still in need of explanation. The magnitude of the problem of visual cell organization is perhaps best illustrated by the phenomenon of visual cell death occurring on a hereditary

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basis in close association with the differentiation of the cell to a functional state, as described below for the C3H mouse. Nevertheless, recent advances in our knowledge of basic properties of the visual cell, especially about the location of enzymes,[11, 12] make it appear that such phenomena are not beyond analysis.

Visual Cell Death from Poisons and ERG Effects

In addition to IAA, oxygen poisoning[13] and X radiation[14] were found to produce visual death in a selective manner; that is, other cells of the retina were unaffected histologically, even when a very large fraction of the visual cell population had been destroyed. Visual cell death occurred acutely in each instance, becoming manifest within twenty four hours after effects upon the ERG had appeared. The histological signs of cell death were the same for all three agents. Pyknosis was the first sign that the cell had been damaged beyond repair (FIGURE 1). Invariably pyknosis was followed within one to two days by chromatolysis; the basophilic substance within the pyknotic mass disappeared, leaving an empty nuclear shell that soon thereafter fragmented and dissolved. Simultaneously, inner and outer limbs underwent swelling, fragmentation and dissolution. Whatever the products of this disintegration may have been, they were readily absorbed, so that within three to six days after the administration of the poison no trace of the visual cell remained. In general, no other tissue replaced the missing layers of the retina.

The vulnerability of the visual cells to IAA differed characteristically over the visual cell population in accordance with the retinal location of the cells and with cell type. In the rabbit, the cells most susceptible to IAA and oxygen poisoning were those of the very center of the retina; susceptibility decreased toward the periphery with about the same gradient as that with which postnatal visual cell differentiation progresses over the retina. Accordingly, the most resistant cells were those located at the ora serrata. In the monkey, the same was true for the rod cells, but the centermost cells—the cone cells of the fovea—were far more resistant to IAA and X radiation of the eye than was any rod cell. (Oxygen poisoning in the rhesus monkey failed to produce retinal changes prior to fatal pulmonary involvement.) The foveal cones were of normal appearance, even when almost all rod cells of the retina had been destroyed.[2, 4] The cone cells outside of the fovea showed atrophic changes that reduced or destroyed their function. Even so, the cells as such survived; that is, the nuclear region of these cells was preserved after outer and inner limb had undergone atrophy. Characteristically, the ellipsoid body of the cones resisted the atrophic changes and moved into a paranuclear position. The atrophy of the peripheral cones was apparently a consequence of the disappearance of the rods and the lack of mechanical support for the cones from the neighboring rods against the forces pressing the retina toward the pigment epithelium.[4] Preservation of the foveal cones was evident even nine months after IAA poisoning, which had resulted in widespread disappearance of the rod cells, including all those close to the central region.[4]

Since IAA and X radiation are unlikely to produce rod cell death by the same mechanism of action, the cone cell in a general way may be safeguarded against irreversible damage better than is the rod cell. Either its metabolic organization is such that these poisons have less chance of killing the cell, or

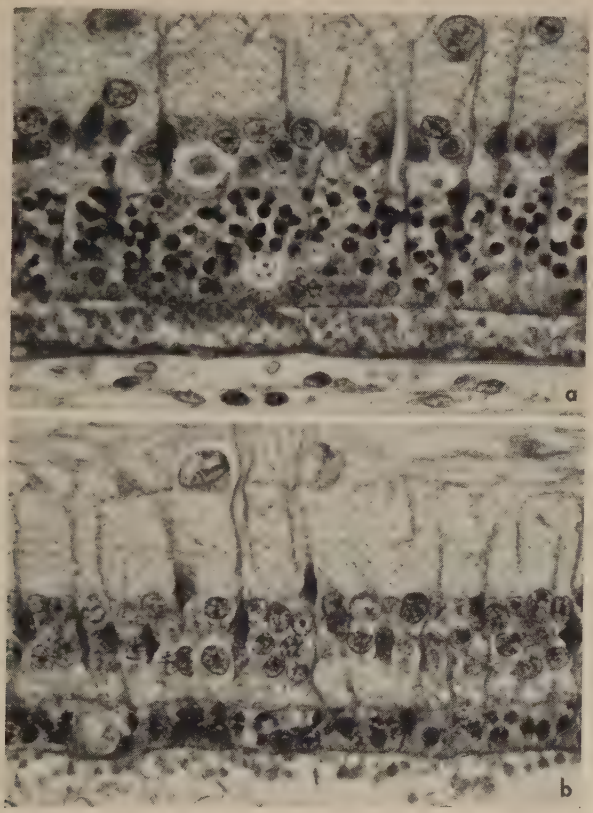

FIGURE 1. Histological signs of selective visual cell death in the rabbit produced by X radiation applied directly to the eye. (a) Typical pyknosis of the visual cell nuclei. Forty eight hours after irradiation many nuclei have already disappeared and lysis inside the pyknotic mass is also evident. The layers of the rods and cones are narrowed and the organelles are deteriorating, the ellipsoid bodies apparently resisting deterioration most strongly. (*b*) The retina four days after X irradiation. The outer nucleic consist of a small fraction of the elements normally present; some nuclei are apparently undamaged, as can be seen by their normal chromatin configuration; the others are pyknotic. A few ellipsoid bodies are visible; they probably belong to cells of which the nucleus appears nonpyknotic. In both sections the cells of the inner layers are normal in appearance and in number. Hematoxylin-eosin stain. Objective (oil) ×50.

its capacity for the rapid repair of a severe damage is greater. The gross morphological difference between cone and rod cell as it exists in the periphery of the retina is neither a likely correlate nor a cause of the greater cone resistance, because the foveal cone cells are very similar in form to the rod cells.

It should be mentioned that differences in vulnerability between cone and rod cells are also evident in hereditary visual cell degeneration. In human retinitis pigmentosa, for instance, visual cell death seems primarily to involve the rod population. This disease, furthermore, spreads over the retina in a manner similar to that with which, in the rhesus monkey, the IAA effect grows over the visual cell population with increasing doses. Curiously, in the rabbit the rod cells of the central retinal region, which are far more susceptible to IAA and oxygen poisoning than the peripheral cells, resist X radiation to a greater degree than do the cells located peripherally. Although we are unable to explain these differences, they all seem to reflect variations in cell properties, rather than in cell environment.

Of the three agents that destroy the visual cells in a selective manner, IAA is the only one for which we can make definite assumptions as to the basic mechanism of its action. The high glycolytic activity of the mammalian retina *in vitro* under aerobic conditions suggests that IAA produces its effect by its capacity to inactivate triose phosphate dehydrogenase, with which it is known to react far more readily than with most other SH enzymes.[15, 16]

In order to show that the *in vivo* effect of a blocking agent is caused by the inhibition of the enzyme system in question it must be proved that the system is actually inhibited *in vivo* and that a quantitative relationship exists between the biological effect and the inhibition of the system. It was found that intravenous doses of IAA that just blocked retinal excitation decreased lactic acid accumulation in the retina 30 to 50 per cent during a subsequent period of ischemia.[4] Furthermore, rabbit retinas removed one-half hour after intravenous injection of the average minimal dose known to damage irreversibly almost the whole visual cell population produced 70 per cent less lactic acid under aerobic conditions in the Warburg vessel than did control retinas.[17] Circumstantial evidence that IAA produces its effect by the inhibition of glycolysis was obtained by comparison of the effectiveness of anoxia and IAA in different species. For instance, the ERG of the frog was found very insensitive to either IAA or anoxia, but failed rapidly when both were applied simultaneously.[1] This is in agreement with the fact that in the frog retina appreciable glycolysis occurs only under anaerobic conditions.[18] Furthermore, ERG components of rabbit and cat that responded very rapidly to anoxia resisted IAA, and those rapidly affected by IAA resisted anoxia—a relationship to be expected if IAA impairs functions depending mainly on glycolysis.

On the basis of these findings the effect of IAA is believed to result from the inhibition of glycolysis. The recent histochemical and electronmicroscopic observations amply support this conclusion. In the visual cell of the rabbit no mitochondria were found anywhere except in the ellipsoid body, which is densely filled with these particles.[8] In the rat, however, an additional single mitochondrion was recently revealed to lie in the terminal rod fiber close to the synaptic ending.[19] Moreover, the measurements by Lowry *et al.*[11] and Schimke[12] of enzymes in the different retinal layers show that the inner limb is extremely rich in enzymes of the respiratory system, for example, malic dehydrogenase and fumarase, whereas the more proximal

part of the cell is abundant in certain glycolytic enzymes. In the monkey, the outer nuclear layer, which contains the nuclei and the perinuclear cytoplasm of the visual cell, is very low in malic dehydrogenase and other related enzymes. In the rabbit malic dehydrogenase was relatively high in this region,[11] but the lack of mitochondria therein, as has been especially reported for the rabbit, renders it very unlikely that a full respiratory system is maintained in this part of the cell. This spatial separation in the visual cell of areas predominantly utilizing glycolysis from the regions where respiration resides should render impairment of the glycolytic systems a far more serious matter than in the case where respiratory energy can compensate at least temporarily for glycolytic inhibition.

Furthermore, it is a highly characteristic phenomenon of the mammalian retina that it resists anoxia to a remarkable degree, in direct contrast to the high susceptibility of the visual cells to IAA. Wegner[20] found that the human retina withstood ischemia, without irreparable damage, for more than thirty minutes. Complete recovery of the rabbit's ERG follows ocular ischemia, even if ischemia is extended for sixty to seventy five minutes.[21] Moreover, death of retinal elements as a consequence of ischemia has been reported to involve first ganglion cells and bipolar cells, whereas visual cell death required longer periods of ischemia.[22, 23] The cytocidal effectiveness of IAA in conjunction with the high capacity of the visual cell to survive anoxia, therefore, implies that a vital function or a vital region of the visual cell derives its energy principally from anaerobic reactions through the Embden-Meyerhof pathway.

The *in vitro* glycolysis of a rabbit's retina, consisting solely of the inner layers after the death of the visual cells following IAA, is not drastically different from that of normal retinas. Its anaerobic glycolysis per milligram dry weight is almost the same as that of control retinas. Aerobic glycolysis is decreased, but by only 25 per cent, when measured in a phosphate-buffered medium (see TABLE 1). Lowry's measurements,[11] moreover, show that in the rabbit—a species that lacks intraretinal capillaries—the enzymes of the glycolytic system are high in the inner layers, whereas malic dehydrogenase is low in comparison with the monkey. Since we have no reason to assume that glycolysis in these layers is less inhibited by IAA than it is in the visual cells, their greater resistance to IAA may be mainly caused by a close relationship within these layers of the glycolytic and the respiratory systems.

IAA very rapidly affected the visual cell function as measured by the ERG, that is, within less than twenty seconds after intravenous injection (FIGURE 2). The excitatory function of the cell, therefore, seems at least as dependent on glycolysis as is cell life. Indeed, the block of visual cell excitation occurred from less inhibition of glycolysis than did the arrest of cell life.[4, 17] Moreover, visual cell death from IAA did not result unless the dose exceeded that needed to reduce markedly the ERG. Otherwise, retinal function recovered.

Details of the relationship between the ERG change and the IAA effect are complicated by remarkable species differences, especially when the course of the IAA effect is compared with that of anoxia or ischemia. This is illustrated for the rabbit and the cat in FIGURE 3. The selective depression

of the b-wave that IAA produces in the rabbit is obtained in the cat with anoxia. In the rabbit the a-wave disappears slowly after the b-wave has been eliminated, whereas in the cat a- and b-waves disappear almost simultaneously and very rapidly. On the other hand, anoxia in the rabbit involves the a-wave prior to a change in the b-wave, provided an intense light stimulus is used.[1] Evidently these species differences concern the order of susceptibility of the functional processes that the ERG measures. They indicate that the organization of metabolism with respect to the maintenance of ERG processes is not established in the same manner throughout the mammalian scale. The finding that a single mitochondrion resides in the

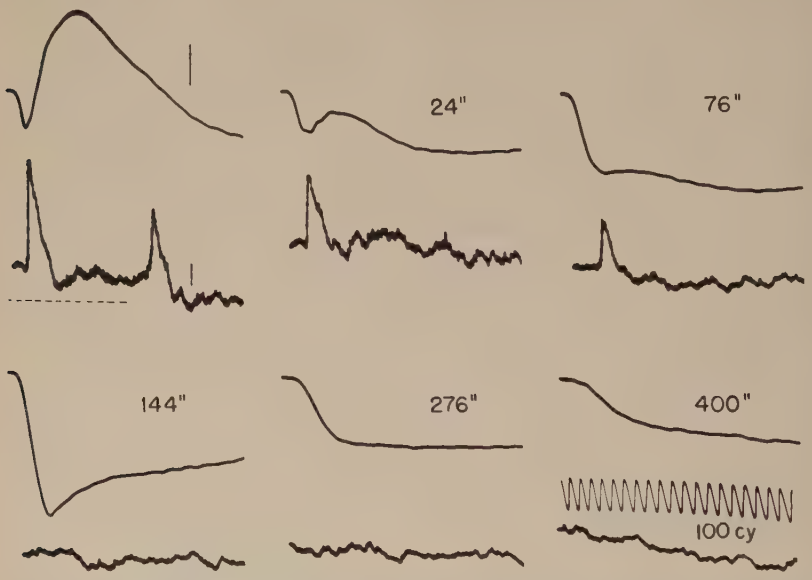

FIGURE 2. Effect of intravenous IAA (15 mg./kg.) on ERG and optic tract potentials in the adult rabbit. The numbers above each tracing denote the time in seconds after the start of injection. The light stimulus was a tungsten light flash of 120 msec. duration.[1]

rat's rod fiber in contrast to the lack of all mitochondria in this region of the rabbit's retina is another example of the great variations in the metabolic organization of the visual cell. Indeed, the ERG effect of IAA in the rat differs from that in the rabbit, but resembles that observed in the cat.[24]

The susceptibility of the visual cells to oxygen poisoning was discovered in the rabbit[13] and seems to apply especially to the retina of this species. In the monkey and the cat, visual cell death from high oxygen pressures was not observed, even when exposure was extended to the longest possible times. In the mouse, visual cell death in a small retinal region resulted from doses in excess of those that destroyed almost the whole visual cell population of the rabbit. However, visual disturbances, for example, marked constriction of the visual field, are known to occur in man prior to manifestation of cerebral involvement at oxygen pressures of several atmospheres.[25, 26]

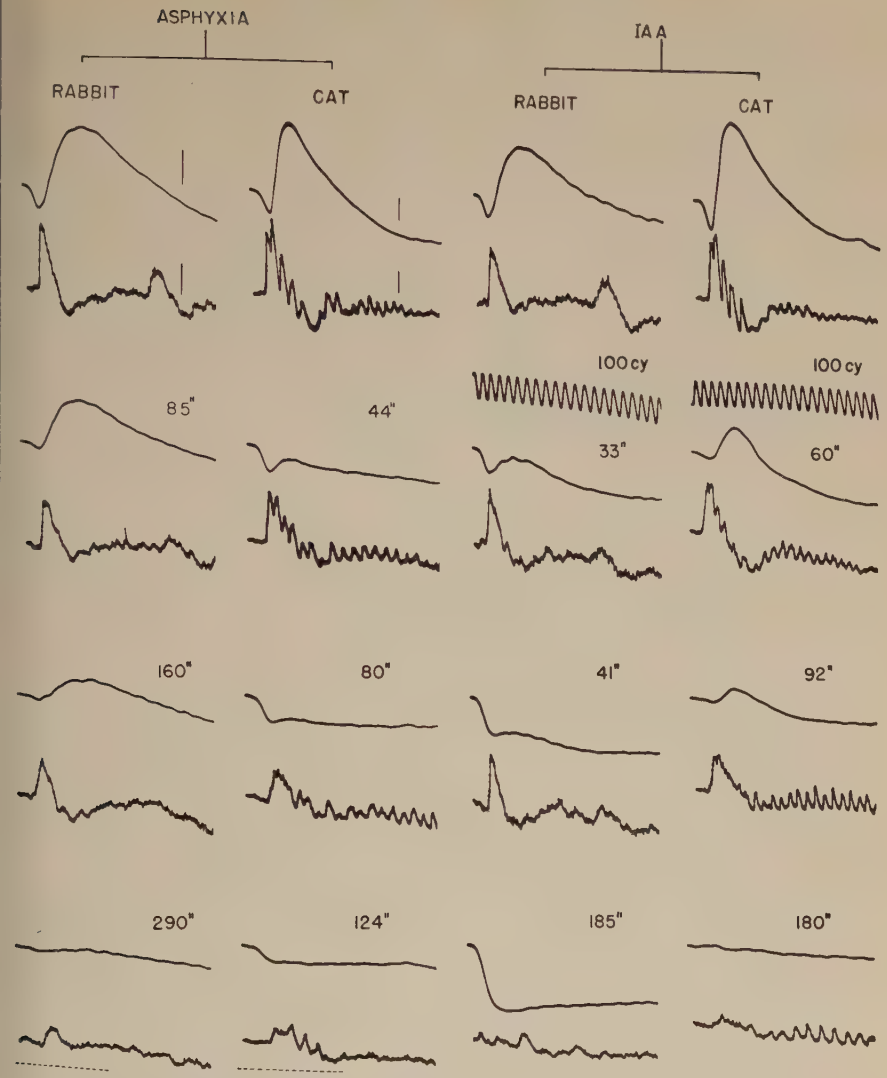

FIGURE 3. Differential effect of anoxia and IAA in rabbit and cat. "Asphyxia" is produced by increasing the intraocular pressure to levels that stop the blood circulation through the eye. The tracings at the left were recorded at the indicated times in seconds after the start of ischemia. Complete recovery from the effects of ischemia occurred. About 1 hour after complete recovery, IAA was administered intravenously (30 mg./kg. in the rabbit, 16 mg./kg. in the cat).

In the rabbit visual cell death resulted from exposures to oxygen concentrations as low as 60 per cent at ambient pressure when the animal was kept in this atmosphere for 5 days. Oxygen concentrations of 90 to 100 per cent at ambient pressure destroyed the majority of the visual cells within 40 to 48 hours. The ERG began to fall after 15 hours of exposure to 100 per cent

oxygen, and the reduction in amplitude progressed slowly during the following 24 hours. Recovery was limited to that fraction of the ERG that had been reduced no more than 8 hours. At oxygen pressures of 7 atmospheres the ERG changed within 20 minutes; exposure for about 100 minutes was required at 3 atmospheres to bring about the same effect. Hence the ERG demonstrated in a unique manner the cumulative character of oxygen poisoning. On the whole, oxygen poisoning is still little understood, despite a new, intriguing hypothesis that it may result from the formation of oxidizing free radicals.[27]

Cell death is an unusual phenomenon of oxygen poisoning. As yet it has been described only for the spermatogenic epithelium.[28] Furthermore, cancer growth and viability are said to be affected,[29] and Warburg[30] recently reported that Ehrlich's ascites cells show depressed glycolysis and viability after long *in vitro* exposure to an oxygen pressure higher than 2½ atmospheres. It may not be mere coincidence that the visual cell resembles cancer and spermatogenic cells in susceptibility to oxygen. The spermatogenic cell, according to Karli,[31] is also sensitive to IAA, and cancer tissue has a very active glycolytic system like that of the retina. However, we have no evidence as yet that oxygen poisoning affects the visual cells by inhibition of glycolysis. A significant summation of the effects of IAA and oxygen poisoning was not observed in tests performed to check this possibility. Undoubtedly, the susceptibility of the rabbit's visual cells to oxygen poisoning reveals an exceptional property of the visual cells.

The most outstanding property of the action of X radiation on the visual cells is a very close association of effects upon the ERG with those upon cell life (W. K. Noell and N. Bailey, "Visual Cell Effects of X Radiation in Rabbit," in preparation, and Bailey and Noell[32]). The lethal cell dose—about 5000 r at 250 kv with single exposure—was only 10 to 20 per cent higher than the dose that produced the slightest ERG effect. To be immediately or rapidly effective upon the ERG, it was necessary to apply doses higher than the cytocidal ones. Cell death was acute, pyknosis developing never later than from 18 to 24 hours after radiation. X radiation, therefore, appears to select for the site of its action a very vital system of the visual cell. In this it differed from all other poisons we studied, including oxygen poisoning. Its action suggests that there is within the visual cell a critical center or system, the impairment of which results in the almost simultaneous loss of cell life and sensory function.

The ERG changes resulting from impairment of retinal metabolism are of different types, as indicated, for instance, by the differences in the effects of anoxia and IAA. The primary effect of a poison upon the ERG may be the selective depression of the b-wave; another poison may first affect the a-wave; and still another poison may produce the more or less simultaneous disappearance of both the a- and the b-waves. The poisons thus seem to select different sites of retinal function for their primary effects, probably in relation to the degree of dependence of the selected site on the particular metabolic process the poison impairs. Although every possible type of ERG change can be observed, most poisons we studied selected the b-wave as their

first or sole target. Such poisons typically reduced the b-wave in amplitude and delayed or retarded its appearance, with the result that the a-wave, with which the b-wave coincides in part or in full, became "unmasked" and appeared with considerably higher amplitude than it had under control conditions (FIGURE 4). This selective b-wave depression was associated with a reduction of retinal excitation as measured, for instance, by the optic nerve response. In addition, the slowing in b-wave appearance was accompanied by a retardation of the optic nerve response (FIGURE 2). The latency of this response could increase two or three times its normal value. On the other hand, no such increase in latency was observed when the a- and b-waves disappeared almost simultaneously.

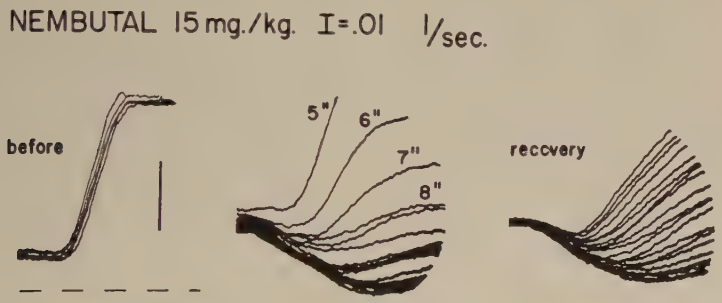

FIGURE 4. Effect of Nembutal on scotopic ERG of the rabbit provoked with a low-intensity stimulus, 200 times threshold. The stimulus, a brief photoflash of a Grass photic stimulator, was applied at a rate of 1 per second. The responses were photographed on the same frame. The tracings of the center picture were obtained at the indicated times in seconds, after the intravenous injection of the drug. The effect begins to appear 5 seconds after the injection. As the b-wave diminishes in amplitude and becomes delayed or slowed in its start, the cornea-negative a-wave develops. The film was moved to a new frame, that shown on the right, about 30 seconds after the injection, when recovery began.

A great variety of poisons produced selective b-wave depression and retardation of retinal excitation. In our experiments these agents were of such diverse kinds as azide, barbiturates (of the sedative or convulsant types), carbon dioxide, trichloroethylene, oxygen poisoning, IAA, and anoxia. Species differences existed for IAA and anoxia, but were not found with azide and barbiturates. Since all these poisons may become effective by impairing cellular metabolism we infer that certain slow processes of visual cell excitation that lead to b-wave generation are the least safeguarded against metabolic impairment.

Differentiation of the Visual Cell to the Functional State

The peculiar organization of the visual cell into compartments of different metabolic activities and the ensuing limitation in viability should develop simultaneously with the anatomical and functional differentiation of the cell. For this reason various aspects of visual cell development during the postnatal period were studied in the rabbit. FIGURE 5 illustrates the course of the histological development in the rabbit between the ages of 2 and 20

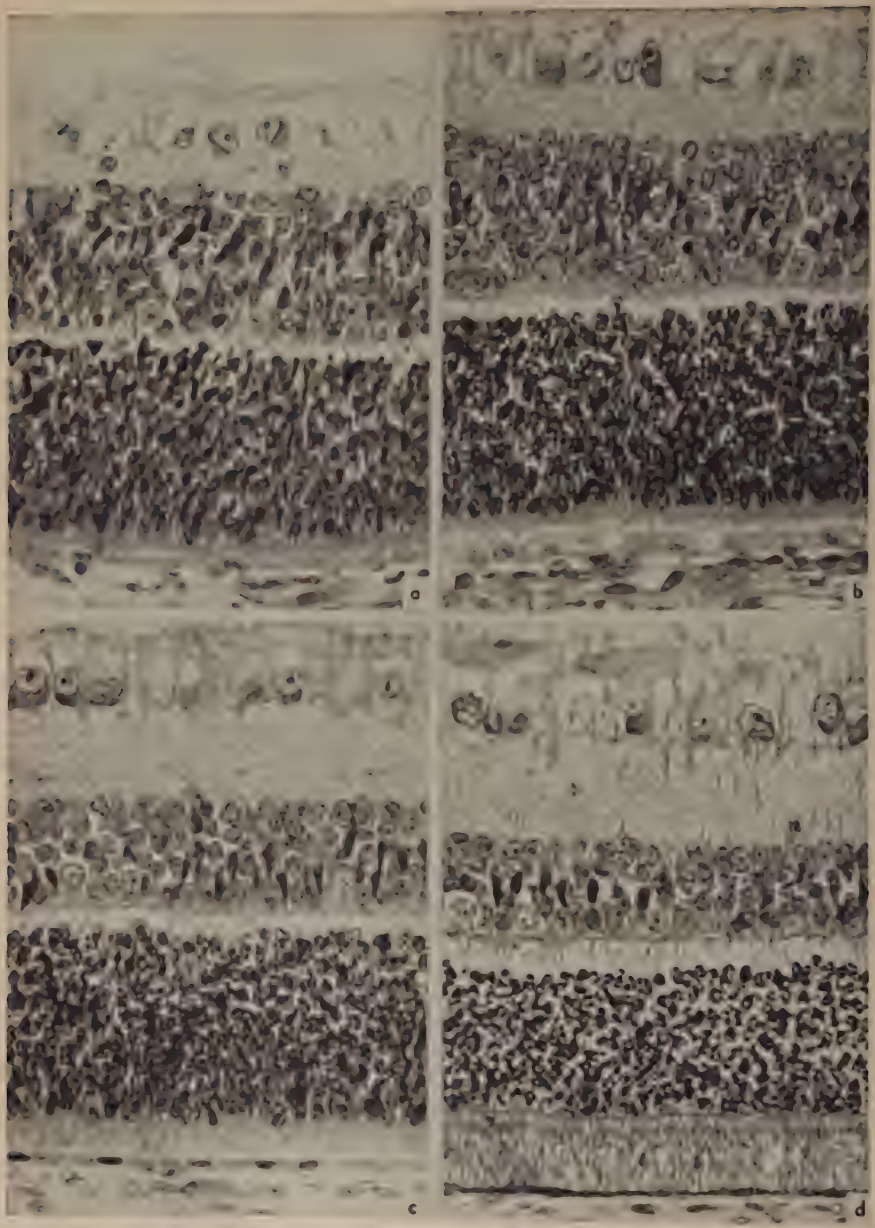

FIGURE 5. Rabbit's retina (a) at the age of 2 days, (b) at 7 days, (c) at 11 days, and (d) at 20 days. Hematoxylin-eosin stain. Objective (oil) ×50.

days. At birth, most visual cells are still in an immature state, but mitotic activity has ceased except for very few cells. The outer nuclei have the immature ovoid form and lack the chromatin fragmentation characteristic of the adult cell with Zenker's fixative. The layer of the "rods and cones" is conspicuously missing, and budding sensory organelles are seen only in the central retinal region. The ganglion cells are already of adult appearance,

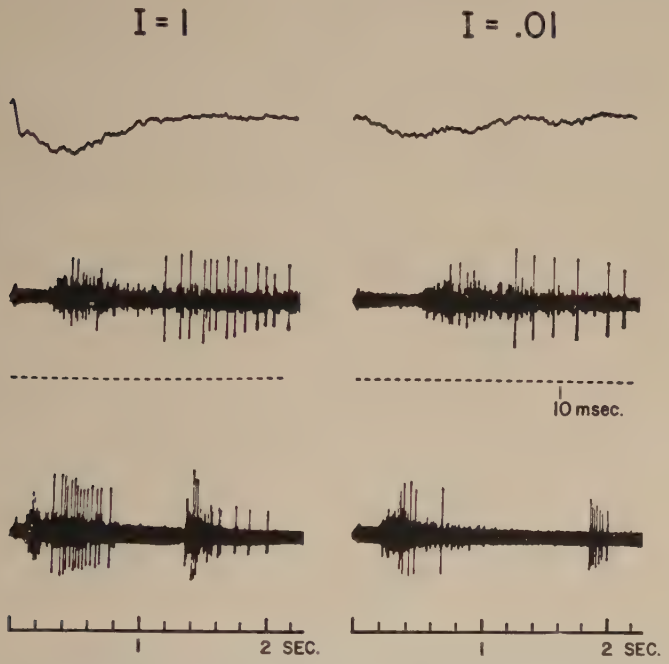

FIGURE 6. Ganglion cell discharges in response to the indicated light intensities (I) at an early stage of retinal functioning—the rabbit at the age of 9 days. A 10-μ platinum electrode was placed at the surface of the central retina after removal of the cornea and part of the lens. Activity appears predominantly in the form of bursts of discharges; excitation apparently alternates with inhibition. The last burst of activity can occur as late as from 5 to 10 seconds after the brief flash of a Grass photic stimulator. Note that the different elements participate to a varying degree in burst activity. The upper and middle row were recorded with the same sweep speed. The ERGs were obtained prior to the insertion of the microelectrode.

whereas many of the bipolar cells are still as immature as the visual cells. After 7 days the bipolar cells have generally reached adult form, and the outer synaptic layer has developed. The sensory organelles are budding all over the retina. They are still very short, but one can now (or a few days later) differentiate between the outer and inner limbs. This development of miniature rods proceeds fastest in the center of the retina, the bulk of the peripheral cells following central development by 1 to 2 days, whereas the cells at the ora serrata are late by 3 to 5 days. The growth of the sensory organelles proceeds with a high rate between the ages of 11 to 18 days, so

that their length is 70 per cent of that of the full-grown ones at about the age of 20 days. Their further growth is slow and occurs in close association with the increase in eye size and the consequent thickening of outer and inner limbs.

The ERG makes its first appearance at about the age of 8 days when the most advanced, central region clearly shows outer and inner limbs. This

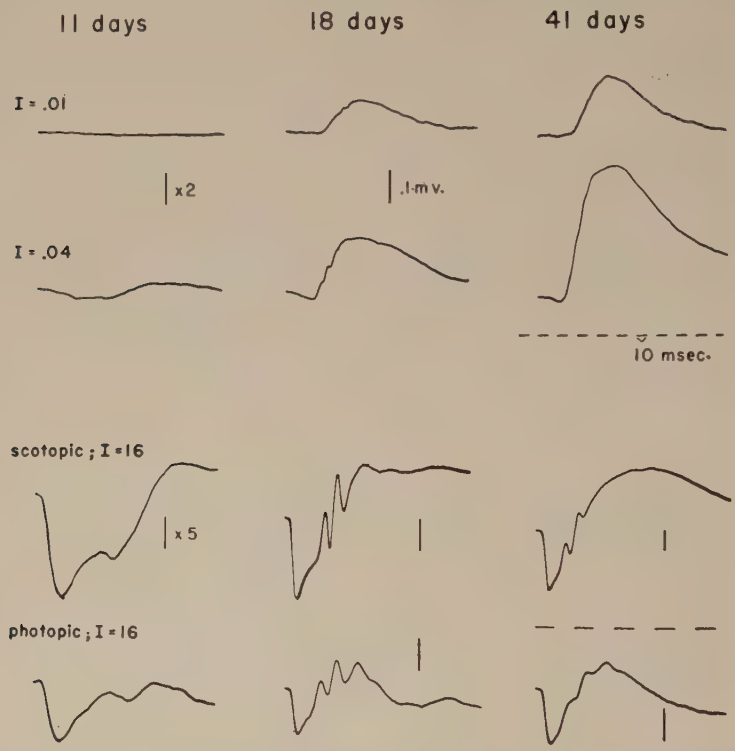

FIGURE 7. Electroretinograms at 3 stages of development. Each column represents 1 experiment; the animals were under Flaxedil and artificially ventilated. The upper 3 rows were recorded under scotopic conditions. Intensity of the light flash is the same in each row. Amplification of the ERGs of the 11-day-old rabbit is twice increased for the records of the 2 top rows and 5 times for those of the 2 lower rows.

early ERG consists mainly of a cornea-negative wave similar in shape to that of the a-wave of the adult ERG after a poison has acutely eliminated the b-wave. As illustrated by the ganglion cell discharges of FIGURE 6, the whole pathway from the visual cell to the ganglion cell seems to function as soon as the ERG has made its appearance. Spontaneous ganglion cell activity has also developed at this time. Furthermore, excitatory as well as inhibitory phenomena in response to illumination are evident. The neural pathways of the retina, therefore, seem to attain a functional state either simultaneously with the development of miniature rods, or slightly earlier.

The b-wave of the ERG appears to a measurable extent at about the age of 10 or 11 days. It rapidly grows during the following days, especially between the twelfth and twentieth days but, when tested under scotopic conditions, it lacks amplitude up to an age of about 90 days (FIGURES 7 and 8). It grows steadily between the twentieth and ninetieth days, during which time its maximal amplitude almost doubles. Simultaneously its scotopic threshold decreases by 1 log unit or slightly more. In contrast to this slow maturation of the scotopic b-waves, photopic responses change little after 20 days. As early as the age of 11 days high-intensity flicker responses are surprisingly well developed (FIGURE 9). At 18 days, moreover,

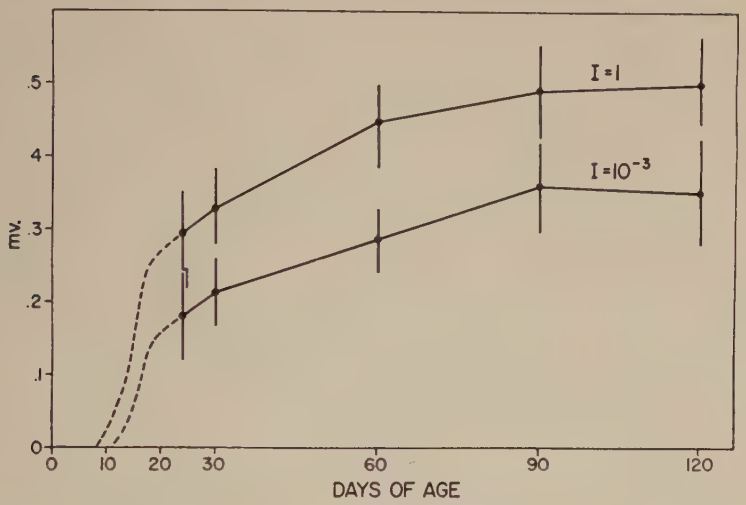

FIGURE 8. B-wave amplitude under scotopic conditions in relation to age at 2 different stimulus intensities. The stimulus was a 120-msec. tungsten light flash. The animals were fully dark-adapted with no general anesthesia and no muscle relaxant. Each point represents the average of 8 to 10 experiments; the vertical lines denote total spread of the measurements. The dotted lines to the left indicate the probable growth of the ERG prior to 25 days, according to measurements obtained with a different technique.

flicker responses are almost the same as they are in older animals. No significant changes in these responses occurred after 30 to 90 days. The same was the case for photopic responses evoked by a single stimulus.

The late increase in the scotopic b-wave may be related to the enlargement of the outer limbs and the increase in rhodopsin content, which one expects to find in association with the increase in outer limb volume. Indeed, the ERGs of young rats on a diet deficient in vitamin A were found to be similar to those recorded during the late phase of retinal development. Just as in the young rabbit, the ERG abnormalities after 3 or 4 weeks on a diet deficient in vitamin A were confined chiefly to scotopic responsiveness (FIGURE 10). The photopic responses were little affected when the average maximal b-wave amplitude had decreased by 30 to 40 per cent. The ERG threshold at this

time was reduced by 1 to 2 log units, that is, slightly more than in the young rabbit at a similar level of scotopic amplitudes in comparison to the adult animal. A decrease of the retinal rhodopsin content by 30 to 40 per cent, but with no histological change, was observed at this stage of vitamin A deficiency.

The late phase of scotopic development also was associated with an increase in the susceptibility of the visual cells to IAA and oxygen poisoning. Neither poison produced visual cell death up to the age of 20 days. Thereafter, the

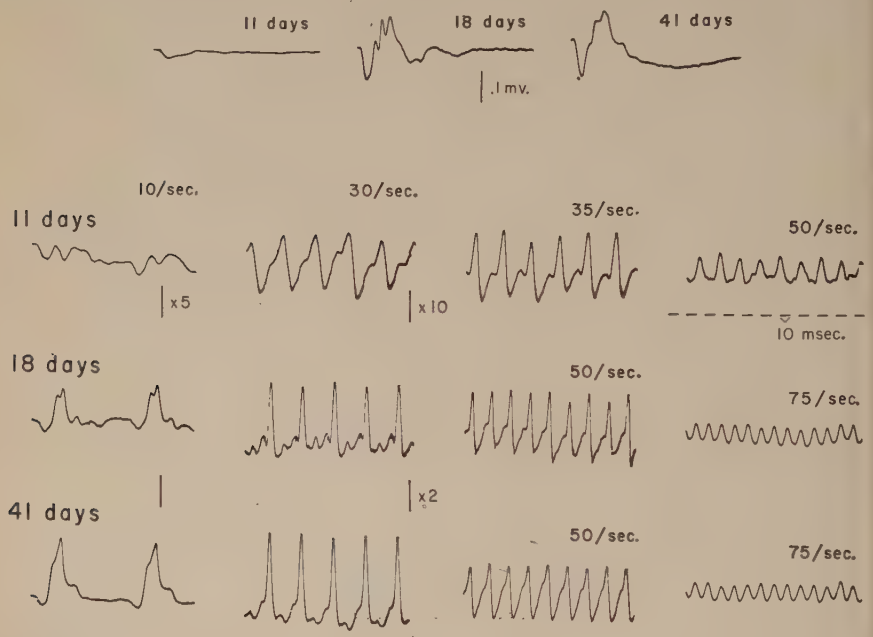

FIGURE 9. Photopic responses at different ages—a continuation of the experiments of FIGURE 7. Topmost row shows the responses to a single strong stimulus at moderate background illumination. All other rows illustrate responses to flicker at different frequencies. At the age of 11 days, a 50/sec. flicker produces almost the same type of response as a 75/sec. flicker at 18 and 41 days. Differences in responsiveness between 18 and 41 days are minimal. Numbers beside calibration marks indicate by what factor the length of the line should be increased in order to represent a signal of 0.1 mv. Amplification at 11 days is 5 times higher than that for the corresponding records at 18 and 41 days.

effectiveness of these poisons became generally apparent and increased to the adult level within the next 2 to 3 months (FIGURE 11). It is improbable that these changes in susceptibility reflect merely differences in blood supply and tissue permeability. Especially for oxygen poisoning, such an explanation seems unlikely because the pressures and exposure times tested exceeded those maximally effective in the adult. If the vulnerability of the visual cells is related, as we believe, to differentiation, then it is not surprising that the immature visual cells escaped the cytocidal effects of these poisons. However, susceptibility increased during a period of time when differentiation

had already progressed very far and when retinal *in vitro* glycolysis and respiration had almost reached adult level. Perhaps during this late stage of retinal development the capacity of the young growing visual cell for the synthesis of essential compounds diminishes, and for this reason cytotoxic agents may more easily produce irreversible damage.

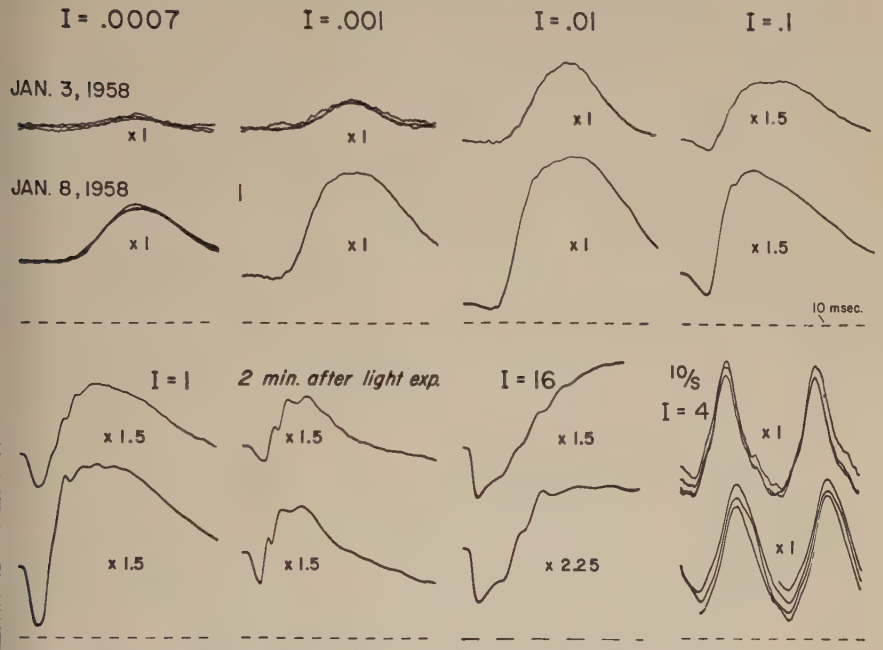

FIGURE 10. ERG of a rat during vitamin A deficiency (Jan. 3), and after high vitamin A doses have been administered for 4 days (Jan. 8). The responses recorded in the top rows and at the left of the bottom rows were obtained after the rat had been in the dark for 14 hours. Scotopic responses were considerably diminished, but the responses to a 10/sec. flicker at a high stimulus intensity do not differ under the 2 conditions. Similarly, the response to a single stimulus (I = 1) 2 minutes after exposure to strong light for a period of 1 minute, is not very different. The tracings at intensity I = 16 were recorded with higher sweep speeds. The numbers beneath the tracings indicate the attenuation of the amplifier system as set for the particular recording.

In Vitro Respiration and Glycolysis of Young and Adult Retinas

Since the gross morphologic differentiation of the visual cells is late in comparison with the differentiation of the inner layers, a study of the changes of retinal metabolism with age was expected to give information on specific metabolic properties of the visual cells. To this end, L. Cohen of our laboratory measured the respiration, glycolysis, and glucose oxidation of the rabbit's retina *in vitro* between the ages of 2 days and several months. Part of the results of this study are summarized in TABLE 1. Average values for the retina at 7 days are compared with those of the adult and, in addition, with those of retinas lacking the visual cells as a result of IAA poisoning 2

to 3 weeks prior to the measurements. These "IAA retinas" from adult animals consisted mainly of the inner layers, and were used to obtain some indication of the metabolic capacities of these layers.

TABLE 1 shows that in all 3 retinas lactic acid production predominates over respiration. The data indicate that this exceptional property of retinal tissue is, in the rabbit, distributed throughout the retinas, and that is is also a phenomenon of the young. The preponderance of glycolysis was evident for retinas at the age of 1 to 2 days, and we may assume that it exists also during fetal life.

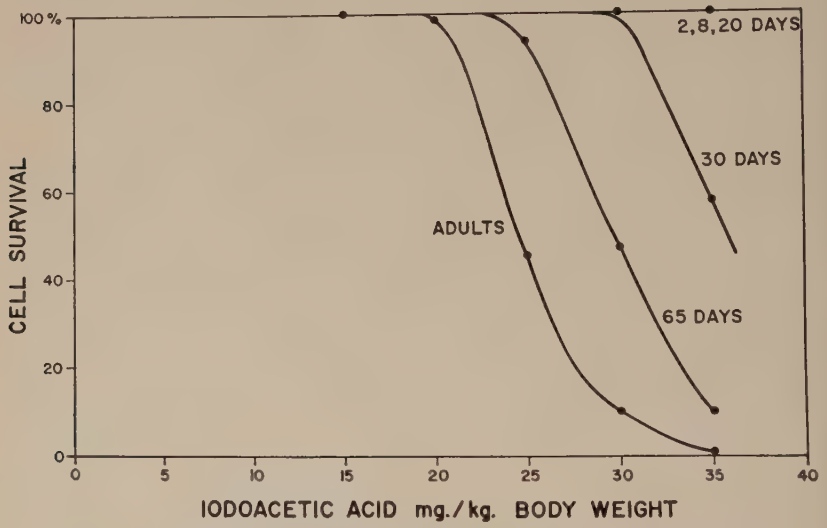

FIGURE 11. Cytocidal effects of IAA at different ages. The graph relates the dose administered to the extent of visual cell survival in a retinal arc from the optic nerve through the ventral region to the ora serrata. Except for 15 mg./kg., the dose was divided into two injections 6 hours apart. The first injection was always 15 mg./kg. Injections were intracardiac in the youngest age groups—2 to 10 days—and intravenous in the others. The systemic toxicity of IAA was greater in the young animals than in the adult.

In Krebs-Ringer phosphate-glucose, the respiration related to dry weight was about 3 times higher in the adult retina than in the retina 2 to 5 days old, or about 5 times per whole retina. About 70 per cent of the increase to the adult level occurred between 12 and 20 days. This is approximately the same period of time during which the outer and inner limbs grow most rapidly, and during which the ERG develops at its fastest rate. The adult retina produced about twice as much lactic acid per mg. dry weight as did the very young one under aerobic conditions, but anaerobic glycolysis was only about 1.6 times higher in the adult. This increase in production of lactic acid occurred earlier than did that of respiration, mainly between 7 and 12 days, when visual cells and neural pathways reached their early functional state.

In addition to having lower metabolic rates, the young retina differed from the normal adult retina in three respects. First, the oxidation of the

TABLE 1*

	Respiration			Glycolysis		Glucose oxidation	
	Q_{O_2} (0.02 M glucose)	Stim. by DNP (0.02 M glucose)	Crab-tree effect	Air Q_L	M.O.Q.	μ Atoms C /mg. dr. wt.	Per cent of oxygen consumed
Seven day....	3.5	2.3×	32%	22	9.3	0.054	35%
Adult.....	9.2	2.5×	10%	43	4.2	0.28	67%
IAA retina....	8.1	—	25%	32	9.0	0.20	55%

* Q values are expressed as μl/hour/mg. dry weight, but were measured during a 10-minute incubation period and approach initial rates. Glycolysis was measured by determination of lactic acid enzymatically, according to the method of Horn and Bruns. Medium: Krebs-Ringer phosphate with or without 0.02 M glucose. The Crabtree effect is expressed as the percentage of inhibition of respiration by glucose. The Pasteur effect in relation to respiration is expressed as the Meyerhof oxidation quotient (M.O.Q.).

$$\text{M.O.Q.} = 3\left(\frac{Q_L{}^{N_2} - Q_L{}^{air}}{Q_{O_2}}\right)$$

glucose of the medium to CO_2, measured with uniformly labeled C^{14} glucose, accounted for only 35 per cent of the oxygen consumed, whereas it accounted for 70 per cent in the normal adult. Consequently, the main substrate for the respiration of the young retina is stored material. Not only does glucose oxidation in the young retina contribute in small part to the respiration, but less than 3 per cent of the carbon dioxide coming from glucose is contributed by carbon-6. It is thus evident that, although the young retina has a very active pathway to produce pyruvate from glucose, as evidenced by high glycolysis, it oxidizes extremely little glucose carbon by way of the tricarboxylic acid cycle.[33] Carbon-1 oxidation, on the other hand, contributes about 20 per cent of the glucose carbon that goes to CO_2, indicating that the hexose monophosphate "shunt" is an important pathway of glucose oxidation. Both the shunt activity and the apparent absence of complete oxidation of pyruvic acid would favor the accumulation of metabolites needed for synthetic purposes. Under the same conditions the adult retina oxidizes far more carbon-6 of glucose than the young one does, but here, too, carbon-1 oxidation is more rapid than that of carbon-6. Although the adult retina oxidizes glucose more effectively than does the young retina, nevertheless, it does not rely solely on the oxidation of the glucose of the medium. In this the young retina seems to resemble the brain more than does the adult one since, as Geiger reports,[34] only 30 to 35 per cent of the respiration of the perfused brain is due to the direct oxidation of glucose in the medium, the remainder of the carbon dioxide coming from stored noncarbohydrate material, which is replenished by synthesis from glucose taken up from the medium.

A second difference between very young and adult retinas is the higher Pasteur effect in the young, as expressed in TABLE 1 by the Meyerhof oxida-

tion quotient. Since IAA retinas show a quotient as high as those of the young, it appears that in the adult retina it is the visual cell that is low in Pasteur effect. Indeed, the data suggest that the visual cells may entirely lack a Pasteur effect.

The third difference between young and adult retinas involves inhibition of respiration by glucose. Cohen[35] found that the young retina respires less when the medium contains physiological concentrations of glucose than when it lacks glucose or any other substrate. This inhibition by glucose amounted to 32 per cent in the 7-day-old retina. It was only 10 per cent in the adult retina, but consistently 25 per cent in the IAA retina (see TABLE 1, the "Crabtree effect"). A similar effect of glucose had been known hitherto only for cancer tissue and was believed to be a special property of cancer metabolism. It was first reported by Crabtree,[36] and has recently been studied extensively with tumor ascites cells.[37-42] According to most of the recent evidence, the Crabtree effect appears to result from a competition between respiration and a high-capacity glycolytic system or a portion thereof, probably for phosphorylative cofactors such as adenosine diphosphate and inorganic phosphate. Glucose, by putting the glycolytic capacity to work, seems to decrease the level of such cofactors, thereby imposing a limitation upon the respiratory chain.

On the basis of this evidence we assume that the Crabtree effect suggests the existence of a competition between respiration and very active glycolytic enzymes in the young retina. This property of the young retina seems to be retained after postnatal development by the inner layers, as suggested by the manifestation of this effect in the IAA retina. However, the slight extent to which this effect is apparent in normal adult tissue, despite its high glycolytic activity, indicates that adult visual cells lack this competition between respiration and glycolysis. The effect measured with the adult retina can be accounted for completely by the magnitude of the effect in the isolated inner layers. In a similar way, the small difference between aerobic and anaerobic glycolysis of the normal adult retina and its low Meyerhof quotient (TABLE 1) suggests that glycolysis and respiration are not intimately related in adult visual cells. It is important to note that the stimulating effect of 2,4-dinitrophenol on respiration was just as high in the adult as it was at 7 days (TABLE 1). It would thus appear that our experimental conditions did not have a selective effect upon the adult retina with respect to the coupling of respiration and phosphorylation, and that the differences measured are not the result of a selective damage of the adult retina by the experimental procedure.

The comparison of the metabolism of the young retina with that of the adult, therefore, leads to the same conclusion drawn from the effect of IAA, namely, that within the visual cell a respiratory system resides together with a high capacity glycolytic system in apparent functional separation.

Hereditary Visual Cell Differentiation in the C3H Mouse

The great effect that differentiation has on the visual cell as a unit appears to be dramatized by the existence of a hereditary condition manifested by the

death of the visual cells during the early phase of their differentiation. This condition was found in the C3H mouse during a search by electroretinography for hereditary visual cell abnormalities. Actually, Dunn[43] had already reported in 1954 that the C3H mouse lacks the visual cells. The C3H abnormality appears to be of the same kind as that studied in albino mice by Tansley,[44] Sorsby *et al.*,[45] and Karli.[31] Probably Keeler's "rodless" mice belong into the same group, although Keeler[46-48] interpreted this abnormality as resulting from arrested development.[49] The condition was present also

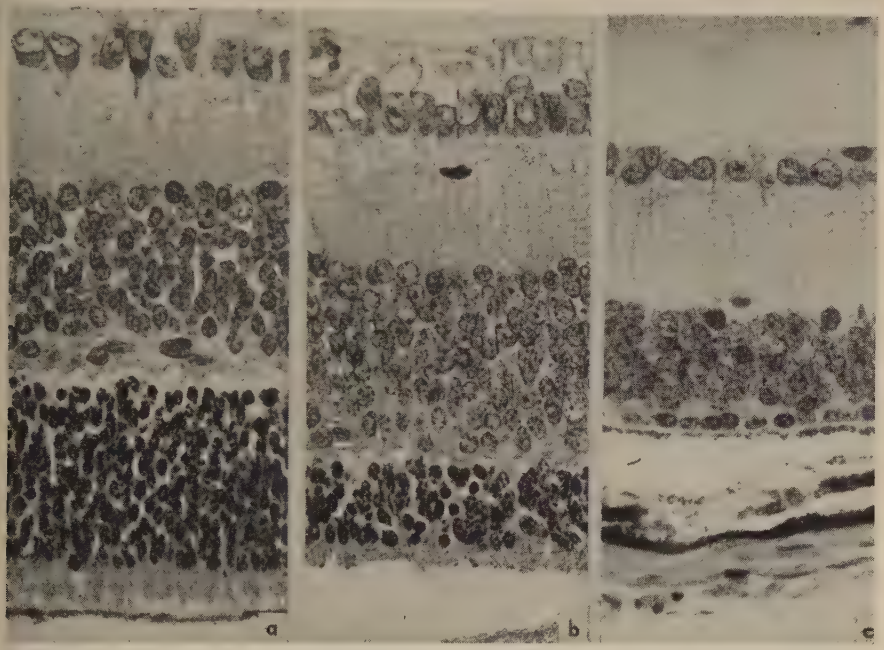

FIGURE 12. Retinas from C3H mice: (a) at the age of 10 days; (b) at 14 days; and (c) at 25 days. At 25 days the eye has increased in diameter and the inner layers have become correspondingly thinner, to the same extent as in normal strains. Hematoxylin-eosin stain. Objective (oil) ×50.

in Swiss mouse populations that we tested. However, because of the great advantages that a pure strain offers, we studied the condition mainly in the C3H mouse. It is inherited as a Mendelian recessive character.

The histological manifestations of this abnormality are illustrated in FIGURE 12. In the C3H mouse early postnatal development of the retina does not differ significantly from that in control strains. At 10 days the C3H and control retinas have advanced to a stage comparable to that of the 8- to 9-day-old rabbit; that is, the inner layers are well differentiated, the outer plexiform layer has been formed, and the sensory organelles have grown to about one fifth of their adult length. Outer and inner limbs are clearly recognizable. However, some differences between C3H and control

retinas are evident at this age: the outer nuclei of C3H are more densely stained; the outer nuclear layer is slightly reduced in width; and the sensory organelles are not as well outlined as in the controls. Moreover, within the central region of the C3H retina a few nuclei are found in a pyknotic state.

During subsequent days, visual cell development progresses through the retinal periphery to the stage of a miniature rod. However, as development proceeds over the retina more and more cells die. Apparently, cell death follows the development of a miniature rod within a few days. At the age of 14 to 15 days, the outer nuclear layer of most of the retina is reduced to about 3 rows of nuclei, and at 17 to 18 days to 1 row. At about 20 days,

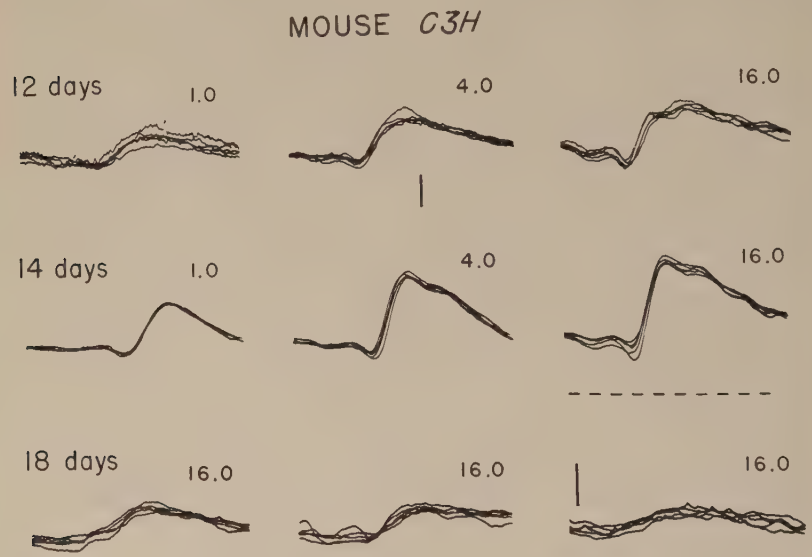

FIGURE 13. Electroretinogram of the C3H mouse at different ages. Each age is represented by a different animal; records of bottom row are from three mice. Amplification is the same, except for the right record of the bottom row.

degeneration has spared only the visual cells close to the ora serrata, and these cells deteriorate within 7 more days. Thereafter the retina uniformly consists of the inner layers, one row of persisting outer nuclei, the outer limiting membrane, and pigment epithelium.

Despite the close association of visual cell differentiation with visual cell death, ERG development occurred. The electric reaction even showed a growth of its amplitude up to about the age of 15 days, and it progressed in form beyond the primitive appearance. It declined as cell death became overwhelming, and it disappeared between 20 and 28 days when the last cells at the ora serrata deteriorated.

In its form, the ERG of the C3H mouse differed from that of a control strain in several ways, as a comparison of FIGURE 13 (C3H) with FIGURE 14 (DBA) will reveal. First, the C3H ERG is lower in a-wave amplitude than

the control ERG, whereas b-wave amplitude may approach control size. Second, the b-wave of the C3H mouse follows the stimulus after a longer time interval than that of controls, but a cornea-negative wave just precedes its emergence, with the result that the ERG distinctly differs from the normal scotopic ERG following low-intensity stimuli. Third, high stimulus intensities are required to elicit the electrical response; in C3H at 12 days the ERG threshold is already about 100 times higher than in DBA.

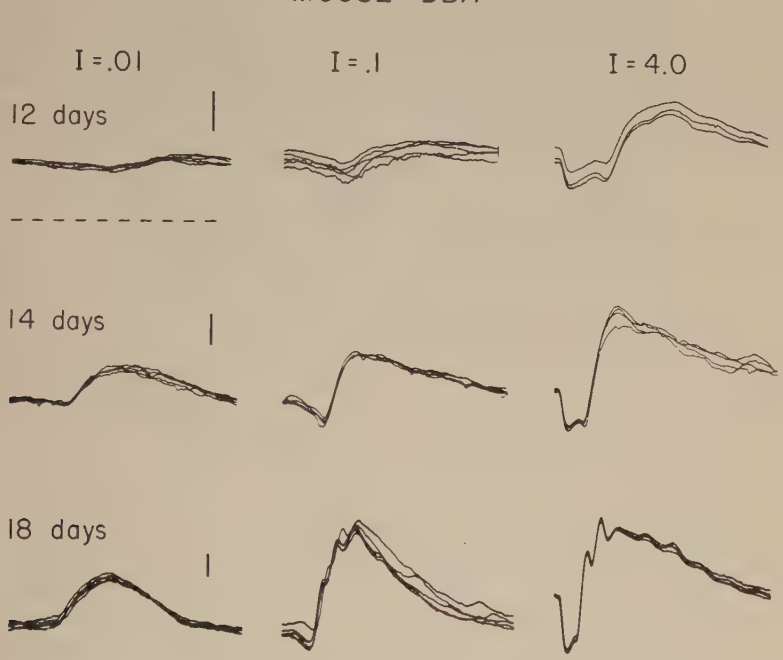

FIGURE 14. Electroretinogram of the normal mouse (DBA). Each age is represented by a different animal. Experimental conditions are the same as for the C3H mouse of FIGURE 13 except that the stimulus intensities cover a lower range.

Occasionally the ERG abnormality of the C3H mouse is of slight degree at the very beginning of retinal function. FIGURE 15 compares early ERGs of C3H mice and C57 Black mice of the same age. The illustrated C3H ERGs are the least abnormal of any litter we recorded; C58 Black was chosen for comparison because ERG development of this strain was generally slower than in other control strains. Compared on this basis, the abnormality of the C3H ERG is very slight, indeed; nevertheless, it can be detected by the slower course of the a-wave, as well as by the delay in b-wave generation at 12 days. It is noteworthy that the a-wave latency of these early C3H ERGs is not prolonged. Similarly, one notes in FIGURE 13 that, despite the prolongation of b-wave latency, the a-wave actually has a short latency, but apparently it consists of two fractions, an early and a late one. The early

fraction of the a-wave may be contributed by elements that have just differentiated to a functional state and do not yet function abnormally to a measurable degree.

The essential abnormalities of the C3H ERG cannot be explained by delayed or arrested development, or by the fact that an increasingly smaller number of elements contributes to the ERG relative to the normal control. On the contrary, the ERGs indicate that the generation of the electric reaction becomes increasingly impaired and abnormal as function progresses beyond the primitive level. Significantly, similar ERG abnormalities were found in the adult rabbit when, after intravenous iodate, outer and inner limbs were undergoing degeneration, which invariably resulted in histological changes similar to those illustrated in FIGURE 16. Generally this degeneration did not produce visual cell death, despite the severe damage of the sensory

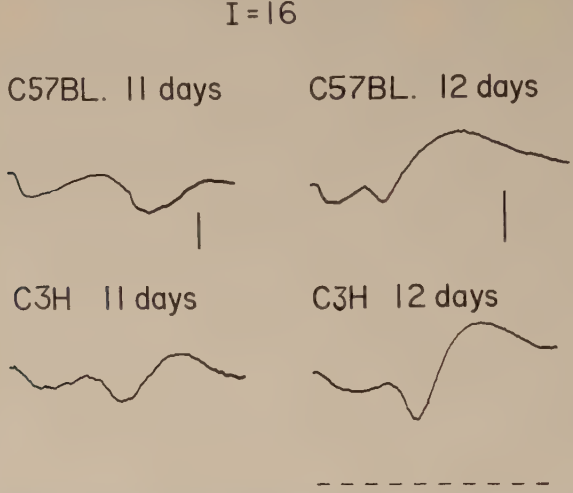

FIGURE 15. Early electroretinograms of the C3H mouse compared with the C57 Black mouse. The records at 12 days were obtained from litter mates of those tested at 11 days.

organelles. This degeneration also occurred as a result of oxygen poisoning and X radiation at doses that were subliminal for the production of visual cell death. After iodate poisoning it was associated with extensive and severe degeneration of the pigment epithelium. No histological signs of pigment epithelial damage were evident in the C3H mouse. ERGs typical for the effect of iodate are illustrated in FIGURE 17. They show the same retardation of the b-wave and a similar preferential reduction of the a-wave as was found in the C3H mouse. The iodate ERG also has a high threshold and its amplitude is markedly reduced.

Since the characteristic ERG abnormalities of the C3H mouse resemble those of the rabbit during sensory organelle degeneration from iodate poisoning, we assume that these abnormalities are manifestations of abnormal sensory organelle function. The actual ERG signs bear this out because they indicate a lesion early in the chain of reactions, which leads to retinal

FIGURE 16. Example for sensory organelle degeneration. The section is obtained from a rabbit 12 days after exposure of the eye to X radiation. Iodate administration leads to the same changes.

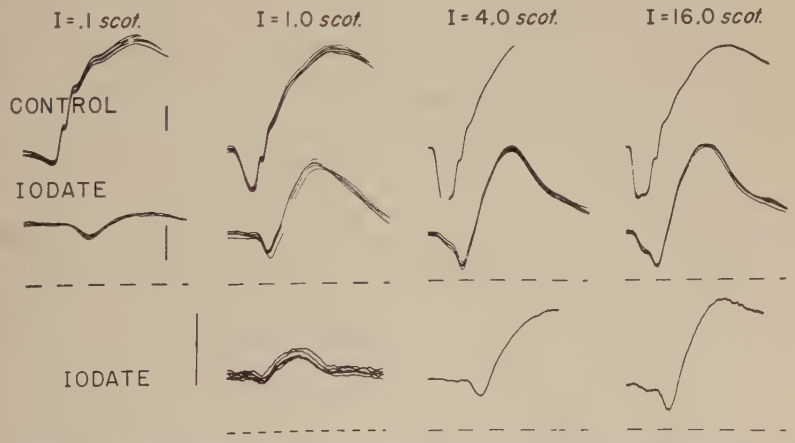

FIGURE 17. Electroretinogram after iodate poisoning. The center record was taken 42 hours after intravenous injection of 5 cc. of a 2 per cent solution of sodium iodate; the bottom record, 3½ days after 4 cc. of 2 per cent sodium iodate. The vertical lines denote 100 μv; each horizontal line represents 10 milliseconds.

excitation in response to light. It may be that from the very beginning the sensory organelle develops abnormally, but the very fact that the sensory function appears shows that the visual cell is able to develop all structures and processes needed for visual excitation. The subsequent death of the cell may be interpreted as the final consequence of a cell abnormality that first

manifests itself in the inability to support normal growth of the sensory organelle. Moreover, the close relationship between cell differentiation and cell death suggests that this abnormality becomes crucial as the cell undergoes the biochemical changes associated with differentiation. To this end the information obtained on the biochemical development of the rabbit's retina may be pertinent, but further studies are needed before such correlations can be attempted. The analysis of this abnormality is probably the most challenging problem related to disorders of the visual system that physiology has ever encountered.

References

1. Noell, W. K. 1951. The effects of iodoacetate on the vertebrate retina. J. Cellular Comp. Physiol. **37**: 283.
2. Noell, W. K. 1952. The impairment of visual cell structure by iodoacetate. J. Cellular Comp. Physiol. **40**: 25.
3. Noell, W. K. 1952. Azide-sensitive potential differences across the eye-bulb. Am. J. Physiol. **170**: 217.
4. Noell, W. K. 1953. Studies on the electrophysiology and the metabolism of the retina. USAF School Aviation Med. Proj. No. 21-1201-0004. Rept. **No. 1.**
5. Sjöstrand, F. S. 1949. An electron microscope study of the retinal rods of the guinea pig eye. J. Cellular Comp. Physiol. **33**: 383.
6. Sjöstrand, F. S. 1953. The ultrastructure of the outer segments of the rods and cones of the eye as revealed by the electron microscope. J. Cellular Comp. Physiol. **42**: 15.
7. De Robertis, E. & C. M. Franchi. 1956. Electron microscope observations on synaptic vesicles in synapses of the retinal rods and cones. J. Biophys. Biochem. Cytol. **2**: 307.
8. De Robertis, E. 1956. Electron microscope observations on the submicroscopic organization of the retinal rods. J. Biophys. Biochem. Cytol. **2**: 319.
9. Wislocki, G. B. & R. L. Sidman. 1954. The chemical morphology of the retina. J. Comp. Neurol. **101**: 53.
10. Sidman, R. L. & G. B. Wislocki. 1954. Histochemical observations on rods and cones in retinas of vertebrates. J. Histochem. and Cytochem. **2**: 413.
11. Lowry, O. H., N. R. Roberts & C. Lewis. 1956. The quantitative histochemistry of the retina. J. Biol. Chem. **220**: 879.
12. Schimke, R. T. 1957. Histochemical determinations of glyceraldehyde phosphate and glycerophosphate dehydrogenases in brain. Federation Proc. **16**: 1428.
13. Noell, W. K. 1955. Visual cell effects of high oxygen pressures. Federation Proc. **14**: 107.
14. Noell, W. K., B. Eichel & P. A. Cibis. 1954. Visual cell and pigment epithelium after high intensity x-radiation. Federation Proc. **13**: 106.
15. Barron, E. S. G. 1951. Thiol groups of biological importance. Advances in Enzymol. **11**: 201.
16. Racker, E. 1955. Mechanism of action and properties of pyridine nucleotide linked enzymes. Physiol. Revs. **35**: 1.
17. Noell, W. K. 1955. Metabolic injuries of the visual cell. Am. J. Ophthalmol. **40**: 60.
18. Oguchi, T. 1952. Über den Stoffwechsel der Netzhaut. II. Der Stoffwechsel der Netzhaut der Reptilien: der Stoffwechsel nach dem Tode des Froschen. Acta Soc. Ophthalmol. Japon. **36**: 1702.
19. Ladman, A. J. & A. J. Mitchel. 1957. Silver staining of mitochondria in rat retina as shown by light and electron microscopy. Stain Technol. **32**: 215.
20. Wegner, W. 1928. Die function der menschlingen Netzhaut bei experimenteller Ischämie retinae. Arch. Augenheilk. **98**: 515.

21. Popp, C. 1955. Die Retinafunktion nach intraocularer Ischämie. Arch. Ophthalmol. Graefe's. **156**: 395.
22. Smith, G. G. & C. D. Baird. 1952. Survival time of retinal cells when deprived of their blood supply by increased intraocular pressure. Am. J. Ophthalmol. **35**: 133.
23. Turnbull, W. 1950. Effects of experimental anaemia in rats. Arch. Ophthalmol. **43**: 9.
24. Noell, W. K. 1951. The site of asphyxial block in mammalian retinae. J. Appl. Physiol. **3**: 498.
25. Behnke, A. R., H. S. Forbes & E. P. Motley. 1935. Circulatory and visual effects of oxygen at 3 atmospheres pressure. Am. J. Physiol. **114**: 436.
26. Donald, K. W. 1947. Oxygen poisoning in Man. Brit. Med. J. **1**: 668.
27. Gerschman, R., D. L. Gilbert, S. W. Nye, P. Dwyer & W. O. Fenn. 1953. Oxygen poisoning and x-irradiation: A mechanism in common. Science. **119**: 623.
28. De Almeida, A. O. 1934. Toxic action of high pressures of oxygen. Compt. rend. soc. biol. **116**: 1225.
29. Stadie, W. C., B. C. Riggs & N. Haugaard. 1944. Oxygen poisoning. Am. J. Med. Sci. **207**: 84.
30. Warburg, O. 1954. Über das Verhalten von Aszites Tumorzellen zu Sauerstoff von höheren Drucken. Arch. Geschwulstforsch. **6**: 7.
31. Karli, P. 1952. Rétines sans cellules visuelles. Arch. Anat. Histol. Embryol. **35**: 1.
32. Baily, N. & W. K. Noell. 1958. Relative biological efficiency of various qualities of x-radiation as determined by the ERG. Radiation Research. In press.
33. Noell, W. K. Differentiation, metabolic organization, and viability. Arch. Ophthalmol. In press.
34. Geiger, A. 1958. Correlation of brain metabolism and function by use of a brain perfusion method *in situ*. Physiol. Revs. **38**: 1.
35. Cohen, L. H. 1957. Glucose inhibition of respiration in the developing retina. Federation Proc. **16**: 165.
36. Crabtree, H. G. 1929. Observations on the carbohydrate metabolism in tumours. Biochem. J. **23**: 536.
37. Brinn, H. & R. W. McKee. 1956. The inhibition of respiration by glucose, fructose and mannose in the Ehrlich mouse ascites tumor. Cancer Research. **16**: 364.
38. Racker, E. 1956. Carbohydrate metabolism in ascites tumor cells. Ann. N. Y. Acad. Sci. **63**(5): 1017.
39. Wu, R. & E. Racker. 1957. Pasteur and Crabtree effects. Federation Proc. **16**: 274.
40. Gatt, S., I. Krimski, & E. Racker. 1956. Reconstructed systems of glycolysis and oxidative phosphorylation. Federation Proc. **15**: 259.
41. Chance, B. & B. Hess. 1956. On the control of metabolism in ascites tumor cell suspension. Ann. N. Y. Acad. Sci. **63**(5): 1008.
42. Bock-Frankenthal, L. & S. Weinhouse. 1957. Metabolism of neoplastic tissue. XII. Effects of glucose concentration on respiration and glycolysis of ascites tumor cells. Cancer Research. **17**: 1082.
43. Dunn, T. B. 1954. The importance of differences in morphology in inbred strains. J. Natl. Cancer Inst. **15**: 573.
44. Tansley, K. 1954. An inherited retinal degeneration in the mouse. J. Heredity. **45**: 123.
45. Sorsby, A., P. C. Koller, M. Attfield, J. B. Davey & D. R. Lucas. 1954. Retinal dystrophy in the mouse: histological and gentic aspects. J. Exptl. Zool. **125**: 171.
46. Keeler, C. E. 1924. The inheritance of a retina abnormality in white mice. Proc. Natl. Acad. Sci. Wash. **10**: 329.
47. Keeler, C. E. 1926. On the occurrence in the house mouse of a mendelizing structural defect of the retina producing blindness. Proc. Natl. Acad. Sci. **12**: 255.
48. Keeler, C. E. 1927. Rodless retina, an ophthalmic mutation in the house mouse. J. Exptl. Zool. **46**: 355.
49. Grüneberg, H. 1943. The Genetics of the Mouse. Cambridge University Press. Cambridge, England.

CENTRIFUGAL INFLUENCE ON THE ELECTRORETINOGRAM*

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Introduction

The existence in the optic nerve of fibers that convey efferent impulses from the higher centers to the retina has been a matter of interest and discussion for many years. Histological evidence for these fibers and for the retinal cellular components of such an efferent system has been provided by Dogiel,[1] Cajal,[2] Johnston,[3] and Polyak.[4] Physiological evidence has been noted by Arey,[5] who discussed the influence of such elements on retinal light and dark adaptation. The importance of such a system in dark adaptation has been mentioned by Pfeifer.[6] Monakow,[7] as a result of animal experiments, described the existence of a centrifugal pathway.

More recently, Polyak,[8] in discussing "the mechanisms of facilitation or inhibition," states: "A structural arrangement whose function seems to be an interference with the synapses between other neurons is exemplified by the horizontal and centrifugal bipolars of the retina . . . the undoubted anatomical relationship mentioned demands an acceptance of some kind of influencing [by the horizontal cells], most likely of the photoreceptor-bipolar relationship." He then infers that these anatomical findings may represent a mechanism for inhibition of rod and cone function, either due to change in threshold or for differential blocking of rods in daylight vision. The centrifugal bipolar cells are considered capable of influencing cone-afferent bipolar transmission.

Granit[9] has been able to inhibit and facilitate spontaneous and evoked retinal potentials by electrical stimulation of the mesencephalic reticular substance, and he considers this to be evidence of centrifugal fibers. Walter[10] has demonstrated efferent fibers terminating in retinal blood vessels.

Motokawa and Ebe[11] have demonstrated that antidromic stimulation of the optic nerve depresses the sensitivity of the retina, and they point out that the electrical response of the retina is dependent on the general condition of the animal. Hartline[12] has demonstrated antidromic inhibition in the eye of *Limulus*. Barany and Hallden[13] showed that central nervous system depressants (alcohol and barbiturates) affect retinal interrelationships.

Dodt[14] found that he could evoke a delayed retinal spike by shocking the contralateral optic tract of a rabbit. He believes this is evidence for the existence of a true centrifugal function.

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In this study an attempt has been made to give further evidence for the existence of centrifugal fibers and to clarify their role.

Methods

Adult cats and Java monkeys were used in this study. The technique on human material is fully described elsewhere.[15]

In cats, tracheal cannulae were introduced under ether anesthesia, and one of the femoral or subclavian veins was cannulated. After all surgical sites had been thoroughly infiltrated with procaine, the animals were permitted to recover and were then paralyzed with succinylcholine or curare.

Artificial respiration and continuous intravenous drip of saline and glucose maintained the preparations for ten to twelve hours without appreciable signs of deterioration. The animal was placed in a prone position in a specially designed head holder and silver-silver chloride, spring-ball electrodes in saline-soaked cotton were gently placed or sutured against the cornea of each eye. In some cases the nictitating membrane was sutured to the inner canthus to avoid interference with the record. A Grass No. 5 polygraph provided direct writing ink records, and a Grass preamplifier P 5R fed a Dumont oscilloscope Type 333 for photographic recording. The tracings obtained were photographically superimposed and printed in sets of five consecutive sweeps.

Acute high-frequency electrical stimulation of the reticular formation was accomplished by suction removal of the medial part of the cerebellum and the positioning of a stimulating electrode posterior to the quadrigeminal bodies, under direct vision. In chronic experiments the electrode was driven stereotaxically through the cerebrum.

For the purpose of unilateral optic nerve section, craniotomies were performed on cats and monkeys, allowing gentle retraction of one of the frontal lobes until the optic chiasm became fully exposed. A length of surgical silk was then looped around one of the optic nerves just distal to the chiasm, and the brain released. While monitoring both electroretinograms (ERG) with a 1/sec. flash delivered to both eyes, the silk loop was sharply pulled, resulting in a section of the optic nerve.

For cross-perfusion experiments,[16] a pair of cats similar in body weight, and age were anesthetized and their left common carotid arteries exposed. Following surgical exposure of the brain stem of the "donor" cat, 10,000 units of heparin sodium were administered to each cat.

After a delay of three hours to allow the heparin sodium to take full effect, a polyethylene tube about 40 cm. long was introduced into the artery of each cat as shown at the left in FIGURE 1. This procedure reduced cerebral blood flow for less than one minute. No appreciable damage to the brain from anoxia is to be expected in this length of time. After both cats were prepared in this fashion, they were placed on a common animal table and connected to a common positive pressure respirator. Either succinylcholine or section of the spinal cord at "C-1" (encéphalé-isolé) paralyzed the animals while we were awaiting their recovery from the ether anesthesia. At this point both polyethylene tubes were cut and cross-connected in the manner shown

at the right of FIGURE 1. Cross circulation was verified after the experiment with the help of dyes.

In the monkey studies similar techniques were used. FIGURE 2 shows an animal in the recording chair with corneal electrodes in place. A Grass H730x8 photostimulator supplied the common light excitation to both eyes.

Since, for all practical purposes, tracings recorded from the cornea and retina are alike when condenser-coupled amplifiers are used (FIGURE 3), the ERG's in this report are those recorded from the corneal surface.

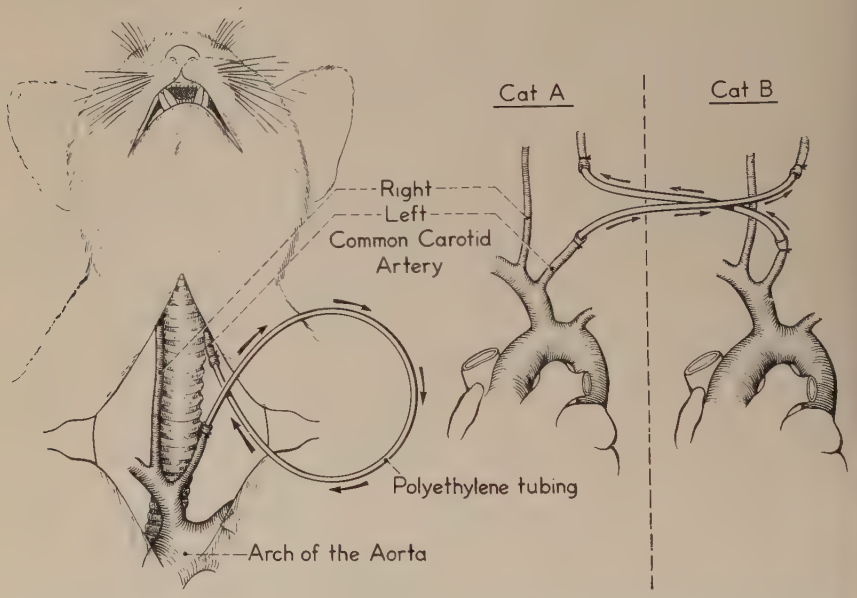

FIGURE 1. Diagram of the cross-perfusion technique. At the left is shown the introduction of the polyethylene tube into the artery; at the right, the manner in which the tubes were connected for cross-circulation between the two cats.

All tracings were performed after a ten-minute period of dark adaptation. Pupils were maximally dilated with atropine drops to eliminate variation in pupil size as a source of error.

Results

An immediate increase in the ERG amplitude followed section of the optic nerve when the animal was under light anesthesia. In deeply depressed animals no increase was noted. Another series of cats was prepared in which unilateral optic nerve section was performed at intervals of time varying from minutes to weeks prior to recording. Drugs were injected into a cannulated vein.

Hexamethonium,* a ganglionic blocking agent, markedly reduced the

* The hexamethonium chloride (Bistrium) used in this study was made avaiable by E. R. Squibb & Sons, New York, N. Y.

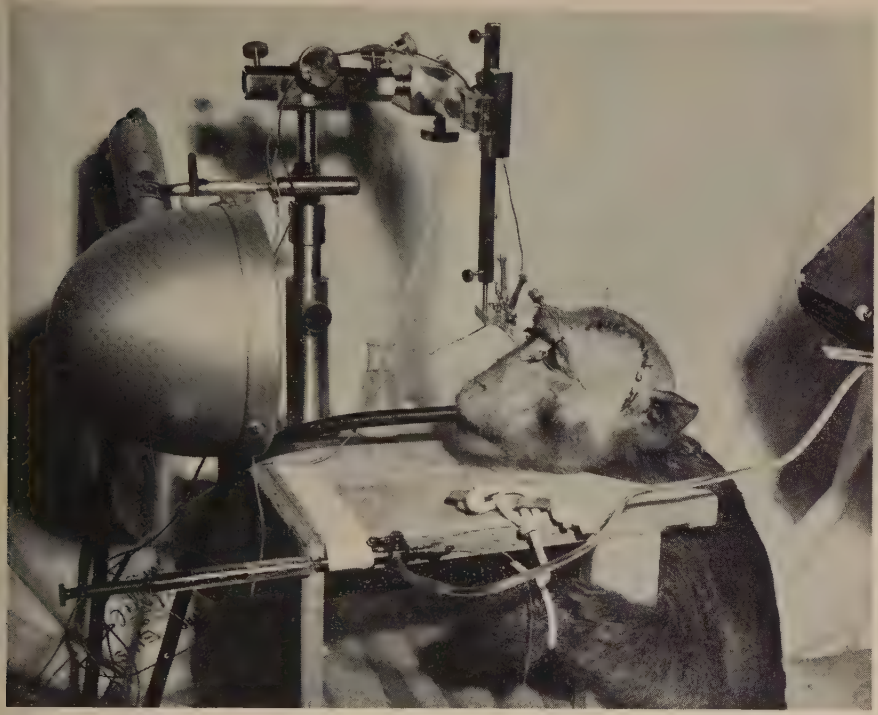

FIGURE 2. Monkey in the recording position with corneal electrodes in place.

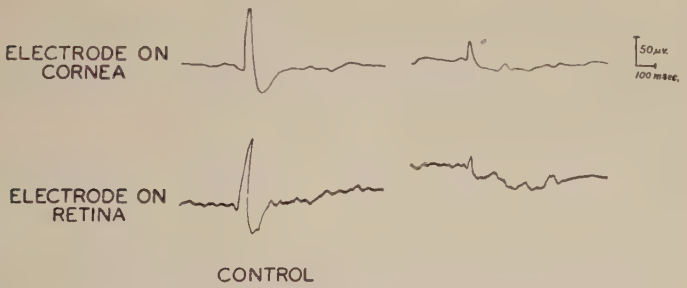

FIGURE 3. Recordings from the corneal and retinal surface, showing their similarity after 10 mg./ion/kg. hexamethonium.

amplitude of the ERG. Strychnine and Metrazol, central nervous system stimulants, also diminished the response (FIGURE 4). This diminution was found only in the eye in which the optic nerve was intact. No change was noted in the eye with the sectioned nerve. Cats having undisturbed optic nerves on both sides reacted as did those having the unilaterally intact eye.

Pentobarbital and ether, central nervous system depressants, caused an increase in the ERG amplitude in the intact eye only; no change was noted in the response on the side of the sectioned nerve (FIGURE 5).

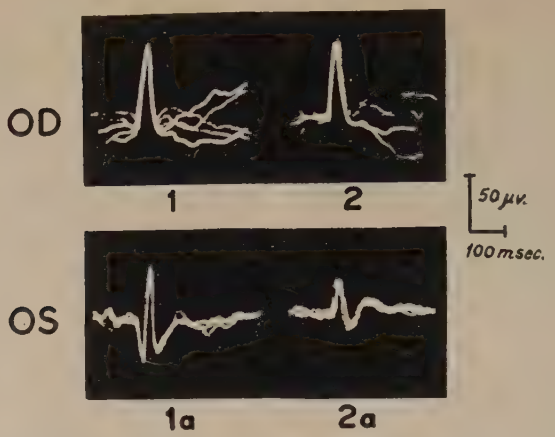

FIGURE 4. Tracings from eyes of cat with optic nerve section just distal to chiasm, OD 2, the right eye; and with optic nerve intact, OS 1a, the left eye. A diminution in the ERG followed intravenous injection of hexamethonium in the left eye only.

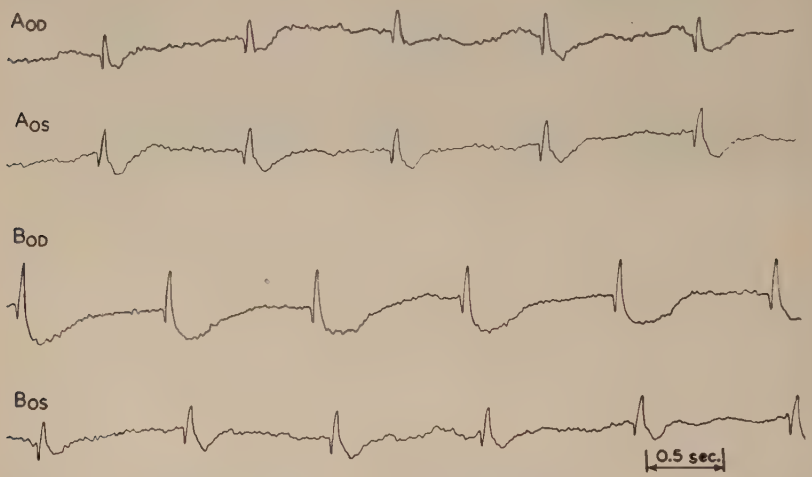

FIGURE 5. ERG's from both eyes of a Java monkey with unilateral optic nerve section on the left (OS), optic nerve intact on the right (OD). An increase in amplitude followed injection of pentobarbital in the right eye only. A is the ERG of the control; B the ERG after 75 mg. pentobarbital injected I.V.

These drugs in varying concentrations were dropped on the surface of the exposed cat and human retina.[15] No significant effect was noted.

The fact that alteration of blood pressure is not the cause of these changes is shown in FIGURE 6. Decreases in blood pressure due to pentobarbital and hexamethonium are about equal but, whereas pentobarbital increases the ERG, hexamethonium decreases it, provided the optic nerves are intact.

It was also interesting to note, as described by Apter and Pfeiffer,[17] that, following the administration of lysergic acid hydroxy butylamide (LSD-25)

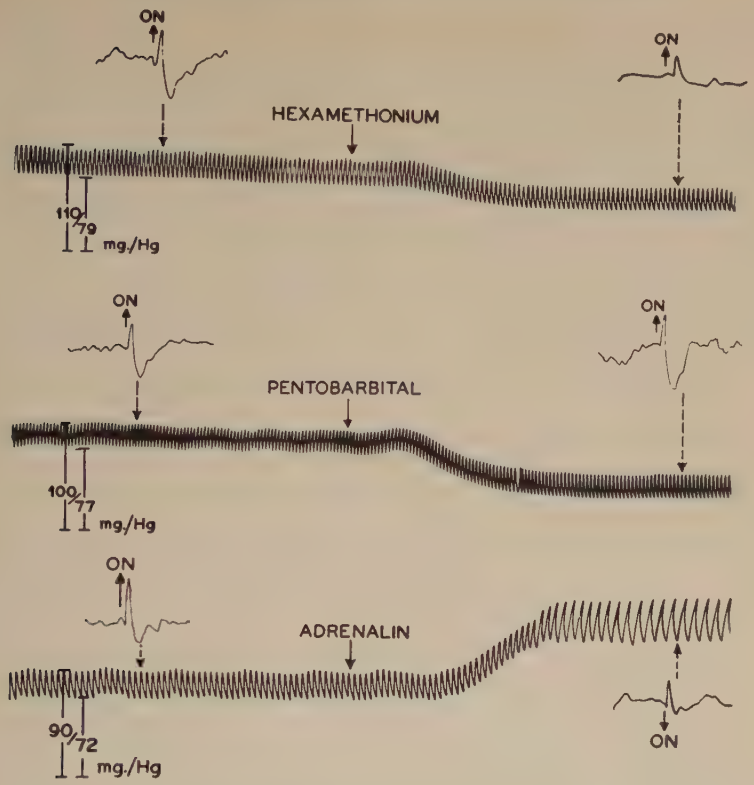

FIGURE 6. Simultaneous recording of blood pressure and ERG in cats, showing that blood-level changes do not correlate with ERG amplitude changes. The ERG diminishes with hexamethonium and epinephrine (Adrenalin) and increases with pentobarbital.

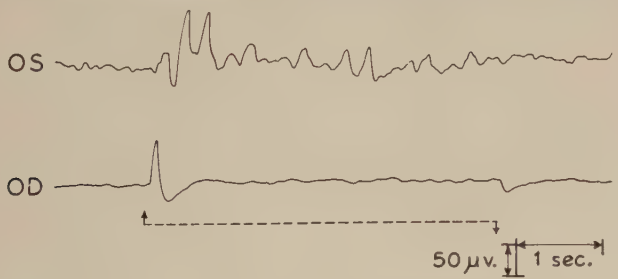

FIGURE 7. Effect of 100 mg./kg. LSD-25 on the ERG of the cat. OS is the left eye; OD is the right eye after section of the nervus opticus.

in cats, spontaneous action potentials occurred occasionally, but only in eyes with intact nerves (FIGURE 7). These findings are described in detail by Evans *et al.*[18] and by J. Jacobson, S. Rosenberg, and G. Gestring (to be published).

FIGURE 8 shows the effect of anesthesia on the ERG. The amplitude

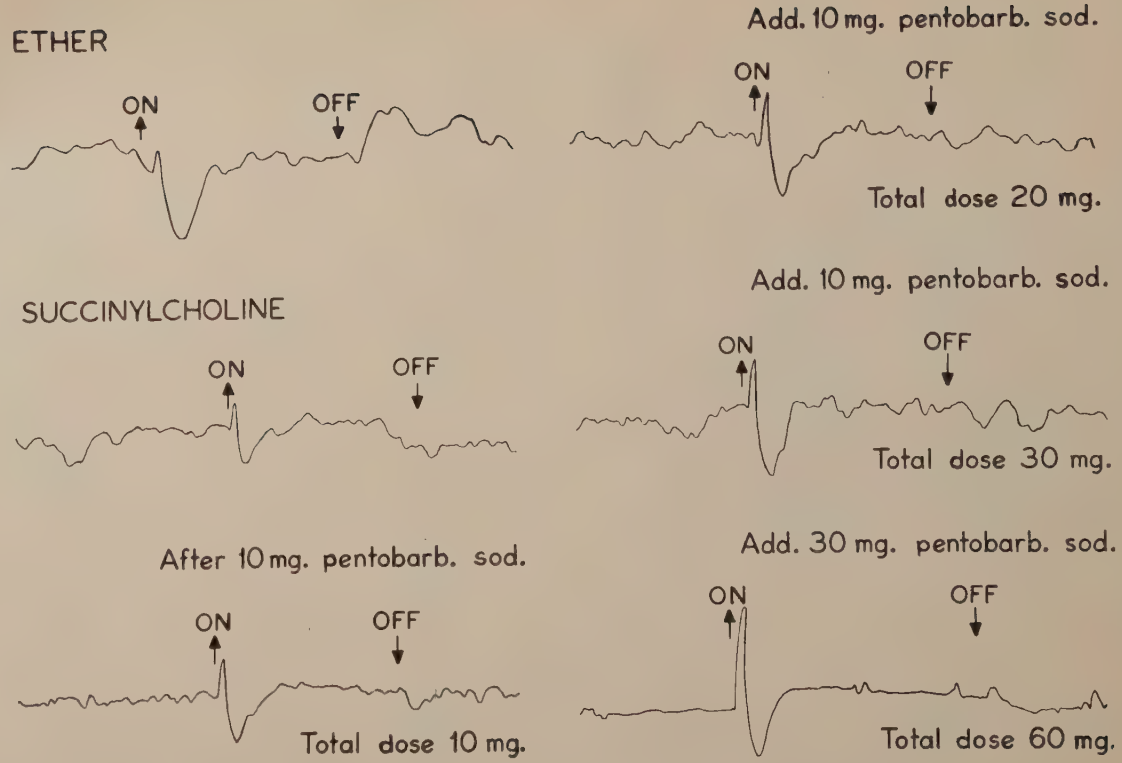

FIGURE 8. Effect of increasing doses of pentobarbital on the ERG of the cat.

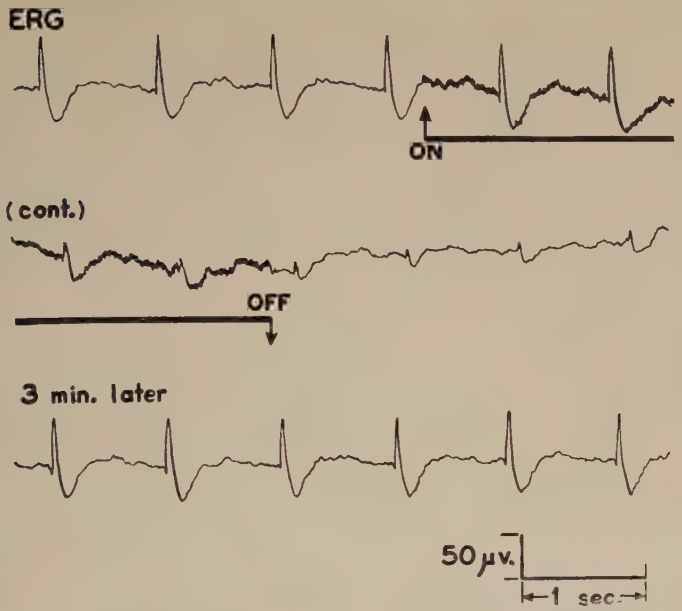

FIGURE 9. Diminution of the amplitude of the ERG of the cat following electric stimulation of the reticular formation.

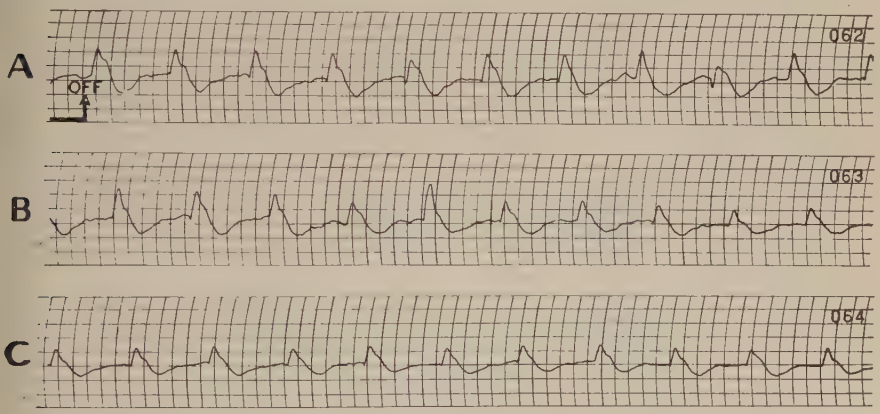

FIGURE 10. ERGs from "recipient" cat of the cross-perfusion study showing diminution, after a delay, in amplitude following electric stimulation of the reticular formation of the "donor" cat.

of the response increases as the level of anesthesia increases. Noell has also noted this finding.*

Electrical high-frequency stimulation of the bulbar reticular formation reduced the ERG amplitude. This effect lasted for over two minutes, although the stimuli bursts never exceeded eight seconds (FIGURE 9).

* W. Noell, 1957. Presented at Symposium on Visual Mechanisms, National Institutes of Health, Washington, D. C. (to be published).

It has been shown that stimulation of this area also inhibits dendritic activity;[19] however, the long-lasting after-effect cannot be explained by cortical neurons inhibiting apical dendrites.

In order to determine the possible role of neurohumors, cross-perfusion experiments were set up. The reticular formation of one animal was stimulated and, while the ERG of the stimulated cat, the donor, diminished immediately, changes in the ERG of the second cat, the recipient, occurred about forty seconds later (FIGURE 10). Intravenous injection of epinephrine (Adrenalin) diminishes the ERG amplitude. This indicates the possibility that epinephrine is a part of the mechanism.

Discussion

The abolition by optic nerve section of the effect of certain drugs on the ERG is evidence for the existence of centrifugal fibers in the optic nerve. In addition, we believe it is possible to hypothesize on the basis of these findings that there exists in the brain a center that controls retinal function, and that we can measure its activity by its effect on the ERG. This center, stimulated physiologically by retinal activity or, as in these experiments, by hexamethonium, central nervous stimulants, or electricity, acts to inhibit retinal activity. Central nervous system depressants reduce the activity of this inhibitory center and thereby increase retinal activity.

Section of the optic nerve interrupts the efferents of the center, causing an immediate increase in the ERG as a result of the lack of central inhibition. Section of the nerve also abolishes the action on the ERG of drugs, since the center, which is the site of action of the drugs, no longer has any way of controlling the ERG.

This type of center is conceived of as a type of feed-back control that regulates the rate and/or intensity of retinal activity and controls dark adaptation.

The cross-perfusion experiments seem to imply the existence of a slower, longer acting neurohumoral system whose action could be on the cephalic center, the retina, or both.

Further experiments utilizing cross-perfused cats with unilateral optic nerve section are now in progress. From these we hope to gain information on the relationship between the neuronal and neurohumoral factors in the visual inhibitory system, while chronic cephalic lesions and stereotaxically implanted electrodes may give us more information on the anatomical location of this inhibitory center.

Summary

(1) The ERG was found to increase in amplitude following optic nerve section or the administration of barbiturates.

(2) The ERG was found to decrease in amplitude following administration of Metrazol, hexamethonium, or electrical reticular stimulation.

(3) Section of the optic nerve eliminated the effects of the drugs.

(4) In cross-perfusion experiments, stimulation of the reticular formation of one animal also produced changes in the ERG of the other animal.

(5) It is felt that these findings are evidence for the existence of centrifugal fibers in the optic nerve.

(6) A hypothetical explanation of the phenomena is offered.

Acknowledgments

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References

1. Dogiel, A. S. 1895. Die Retina der Vogel. Arch. mikrobiol. Anat. u. Entwicklungsmech. **44**: 622.
2. Ramon y Cajal, S. 1909. Histologie du système nerveux de l'homme et des vertébrés. Maloine. Paris, France.
3. Johnston, J. B. 1906. The Nervous System of Vertebrates. Blakiston. Philadelphia, Pa.
4. Polyak, S. 1957. The Vertebrate Visual System. : 246–255; 579; 589. Univ. Chicago Press. Chicago, Ill.
5. Arey, L. 1916. The function of the efferent fibers of the optic nerve of fishes. J. Comp. Neurol. **26**: 213.
6. Pfeifer, R. 1930. Die nervosen Verbindungen des Auges mit dem Zentralorgan. *In* F. Schieck & A. Bruckner. Kurzes Handbuch der Ophthalmologie. pp. 396. Springer. Berlin, Germany.
7. Monakow, C. V. 1889. Experimentelle und Pathologisch-Anatomische Untersuchungen die Optischen Centren und Bahnen. Arch. Psychiat. Nervenkrankh. **20**: 714.
8. Polyak, S. 1957. The Vertebrate Visual System. : 579–580. Univ. Chicago Press. Chicago, Ill.
9. Granit, R. 1955. Centrifugal and antidromic effects on ganglion cells of retina. J. Neurophys. **18**: 388.
10. Walter, J. R. 1957. Über Endigungen Zentrifugaler Nervenfassern an dem Blutgefassern der menschlichen Netzhaut. Arch. Ophthalmol. Graefe's. **6**: 158.
11. Motokawa, K. & M. Ebe. 1954. Antidromic stimulation of optic nerve and photosensitivity of cat retina. J. Neurophysiol. **17**: 364.
12. Hartline, H. 1953. Mutual inhibition among the receptors of the eye of *Limulus*. Proc. 19th Intern. Phys. Congr. Montreal, Canada. : 441.
13. Barany, E. & U. Hallden. 1947. The influence of some central nervous depressants on the reciprocal inhibition between the two retinae as manifested in retinal rivalry. Acta Physiol. Scand. **14**: 296.
14. Dodt, E. 1956. Centrifugal impulses in rabbit's retina. J. Neurophysiol. **19**: 301.
15. Jacobson, J., G. Stephens & G. Gestring. 1958. Electrical activity of excised human retina. A. M. A. Arch. Ophthalmol. In press.
16. Purpura, D. 1956. Observations on a neurohumoral mechanism of reticulocortical activation. Am. J. Phys. **186**: 250.
17. Apter, J. & C. Pfeiffer. 1957. The effect of the hallucinogenic drugs LSD-25 and mescaline on the electroretinogram. Ann. N. Y. Acad. Sci. **66**(3): 508.
18. Evans, L., J. Jacobson & G. Gestring. 1958. Anat. Record. In press.
19. Purpura, D. 1956. Observations on the cortical mechanism of reticulo-cortical activation accompanying behavioral arousal. Science. **123**: 804.

HUMAN RETINAL RESPONSES*

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The human eye appears at first glance to be a most unpromising organ from which to try to obtain electrical records of the process of photoreception. As other papers in this monograph have shown, the eyes of other animals can be excised, dissected, drugged, or probed with microelectrodes. Furthermore, relatively simple eyes can be studied, which minimizes the complexity of the responding mechanisms. With the human eye, however, we are in the position of trying to analyze the most complex of visual responses by remote means of recording, as I have discussed elsewhere.[1] This paper indicates the difficulties and limitations of recording from the human eye and then describes the progress that has been made in overcoming them.

A major difficulty in the past has been the discomfort or injury involved in attaching electrodes to the eyeball. It is true that in animal preparations action potentials have long been recorded by the use of external wick electrodes. But it has been necessary to immobilize the animal in order to maintain a stable electrical contact between a corneal electrode and a reference electrode elsewhere on the head. When that is done, the electrodes can be used to record an electroretinogram (ERG) in response to a flash of light. Control experiments have shown that the ERG arises mainly from the retina of the eye. Human ERGs have also been recorded in this way, notably by Sachs,[2] Cooper *et al.*,[3] Hartline,[4] and Adrian.[5]

The procedure is a heroic one, however, because the most easily accessible region of the eye, the cornea, is a region that is also most richly supplied with pain receptors. Although a local anesthetic can be applied to the cornea, the eye can never be held completely still, even during attempted steady fixation. Thus, only intermittent contact with the human cornea can be achieved by the use of a wick electrode.

A second difficulty with ERG work on the human eye is imposed by the complexity of the eye itself. The presence of both photopic and scotopic systems in a retinal structure displaying elaborate spatial and temporal interactions almost guarantees that the electrical responses will be difficult to analyze and interpret. All the various forms of rod and cone receptor are stimulated by the intense flashes of light that are necessarily used in these experiments. Recent animal experiments with microelectrodes, particularly those of Tomita and Funaishi,[6] Tomita and Torihama,[7] Svaetichin,[8] and Brindley,[9] have demonstrated that the various components of the ERG originate within different layers of the retina. The structures involved appear to be primarily the receptors and bipolar cells. We may therefore assume that the human ERG indeed represents a multiplicity of responses in which it is difficult to isolate the separate contributions of the various retinal elements.

* The research for this paper was supported by a research contract from the Office of Naval Research, Washington, D. C.

A third difficulty is that no measurable ERG is produced unless the stimulating light is of sufficient magnitude to affect large masses of cells. Area and intensity of stimulation are reciprocally related; for that reason, if it were possible to confine a flash of light to a small spot on the retina, no measurable response would arise until a very high stimulus intensity was reached. At this high level, however, the light scattered throughout the retina becomes so effective that almost the entire ERG is found to originate in the nonfocal area.[10, 11] Thus it is practically impossible to record electrical responses, for example, from the rod-free area of the outer fovea.[5] Indeed, a beam of light concentrated on the blind spot scatters so widely over the peripheral retina that the resulting ERG is nearly indistinguishable from one elicited by a beam that is directed on a region containing the normal complement of rods and cones.[12, 13]

One more limitation should be mentioned, and this is the fact that the responses of the photopic visual systems are largely overshadowed by those of the scotopic.[14] This may be a consequence of the fact that in the human eye the rods outnumber the cones by a ratio of about 20:1. Clearly the ERG but poorly registers the activity that is basic for the superb color vision and form perception that we know to be mediated by the human eye.

Having noted the rather formidable difficulties that have plagued experimenters on the human ERG, let us now turn to some of the encouraging progress that has recently been made in reducing or eliminating them. One outstanding problem, that of the electrode, was largely eliminated when plastic contact lenses became available during the early 1940s. A metallic electrode sealed into such a lens permits a firm but comfortable attachment of an electrode to the cornea.[15] A reference electrode may be placed on the skin of the forehead. The human ERG is now routinely recorded between such electrodes for both experimental and clinical work, and the consistency of responses from a co-operative human subject rivals that of an excised animal eye.

Some of the other difficulties mentioned above cannot be overcome so readily. The complexity of the retina and the scattering of light are inherent properties of the human eye, and we must learn to live with them and to understand them partly in terms of animal experiments. We have learned, for example, that the first portion of the ERG is a negative "a-wave" of extremely short latency that appears to originate at or near the receptor cells. This a-wave is itself complex in nature, portions of it appearing to be photopic and scotopic in their origin.[16] The second prominent feature of the ERG is the positive b-wave; but this is most certainly composed also of photopic and scotopic components. It is believed by several investigators to have an origin different from the a-wave, probably further upstream from the photoreceptors.[17, 18] An early positive component prominent in response to red light is the x-wave of Motokawa and Mita.[19] This early positive wave, described by Adrian[5] as a photopic b-wave, has been shown to be lacking in red-blind subjects, that is, protanopes.[20, 21] Slow portions of the b-wave develop over the course of dark adaptation[22] and are found to be greatly reduced in size among congenitally night-blind individuals.[23, 24] Large

scotopic b-waves are readily elicited by stimuli that are known to be most effective for human rod vision, that is, by light of about 500-mμ wave length shining upon a large retinal area with flash durations of 100 msec. or longer.

There is a still slower positive response, the c-wave, that sometimes appears in the human ERG. When this response appears with a latency of from 0.5 to 0.8 sec., however, it can be shown to arise not so much from the retina as from reflex responses of the intraocular muscles. This is attested by the fact that the magnitude of the effect is reduced or abolished by the action of atropine.[25] Off-effects are not prominent in the human ERG, but appear reliably under certain conditions of light adaptation.[26, 27] The human ERG, then, complex though it may be, can be analyzed, and its components related to some of the photopic and scotopic mechanisms of vision.*

Most recently a great deal of progress has been made in recording the photopic components of the ERG. Several specific techniques have been developed for overcoming the last of the limitations enumerated above, namely the overshadowing of photopic by scotopic responses. Among these techniques are the use of flickering light of moderately high frequency, to which the scotopic system is too slow to respond;[5, 28] the use of red light to minimize the stimulation of the rod receptors;[5] the use of flashes of less than 0.04 sec. duration, well below the critical duration of the rods;[29, 30] the use of large-area central stimulation to bring in large numbers of cone receptors;[31] and the use of various techniques of selective amplification.[32]

The flicker method has already yielded spectral sensitivity functions closely approximating the psychophysical ones, provided that sufficiently large stimulus fields are employed.[33] The flicker method is also a convenient one for clinical use. Dodt and Wadensten[34] and Iser and Goodman,[35] for example, have emphasized the necessity for flicker records along with single-flash records for a proper assessment of retinal function.

Recently Henkes *et al.*[32] have made use of selective amplification to accentuate the responses to flickering light. This applies a principle first suggested by Granit and Wirth,[36] namely, the use of a frequency analyzer in the output of the amplified ERG to measure responses of minimal size. An alternative scheme that we have recently employed is to sweep a frequency analyzer through a small range of frequencies from slightly above to slightly below the frequency of the stimulating light. This permits an easy comparison between energy at the precise frequency of the flickering light and energy to either side, thus ensuring that that actual response to the flickering light has been measured.

Conclusion

The human ERG can now be recorded with considerable precision under good experimental conditions. The form of the response is a complex one,

* The existence of both fast and slow components in the ERG makes it imperative to use appropriate techniques of amplification and recording. Direct-coupled amplification and high-speed oscillographic recording are required for any serious investigation of the wave form of the ERG.

but it is in many cases sufficiently well understood to be used as an indicator of scotopic and photopic activity. Thus the ERG can be used as an objective measure to supplement the information supplied by psychophysical methods on human dark adaptation, spectral sensitivity, brightness discrimination, and flicker. The ERG has taken its place among clinical diagnostic procedures. Reflecting as it does the functioning of retinal structures at or near the receptors, it now is used to indicate the presence and degree of defects occurring at this level.

References

1. Riggs, L. A. The human electroretinogram. A.M.A. Arch. Ophthalmol. To be published.
2. Sachs, E. 1929. Die Aktionsströme des menschlichen Auges, ihre Beziehung zu Reiz und Empfindung. Klin. Wochschr. **8**: 136.
3. Cooper, S., R. S. Creed & R. Granit. 1933. Note on the retinal action potential of the human eye. J. Physiol. **79**: 185.
4. Hartline, H. K. 1925. The electrical response to illumination of the eye in intact animals, including the human subject and in decerebrate preparations. Am. J. Physiol. **73**: 600.
5. Adrian, E. D. 1945. The electric response of the human eye. J. Physiol. **104**: 84.
6. Tomita, T. & A. Funaishi. 1952. Studies on intraretinal action potential with low-resistance microelectrode. J. Neurophysiol. **15**: 75–84.
7. Tomita, T. & Y. Torihama. 1956. Further study on the intraretinal action potentials and on the site of ERG generation. Japan. J. Physiol. **6**: 118.
8. Svaetichin, G. 1956. IV. Notes on the ERG analysis. Acta Physiol. Scand. **39**: 55.
9. Brindley, G. S. 1956. Responses to illumination recorded by microelectrodes from the frog's retina. J. Physiol. **134**: 360.
10. Boynton, R. M. 1953. Stray light and the human electroretinogram. J. Opt. Soc. Am. **43**: 442.
11. Crampton, G. H. 1956. The effect of a glaring light source on the human electroretinogram. J. Comp. and Physiol. Psychol. **49**: 534.
12. Boynton, R. M. & L. A. Riggs. 1951. The effect of stimulus area and intensity upon the human retinal response. J. Exptl. Psychol. **42**: 217.
13. Asher, H. 1951. The electroretinogram of the blind spot. J. Physiol. **112**: 40.
14. Riggs, L. A., R. N. Berry & M. Wayner. 1949. A comparison of electrical and psychophysical determinations of the spectral sensitivity of the human eye. J. Opt. Soc. Am. **39**: 427.
15. Riggs, L. A. 1941. Continuous and reproducible records of the electrical activity of the human retina. Proc. Soc. Exptl. Biol. Med. **48**: 204.
16. Armington, J. C., E. P. Johnson & L. A. Riggs. 1952. The scotopic a-wave in the electrical response of the human retina. J. Physiol. **118**: 289.
17. Granit, R. 1933. The components of the retinal action potential and their relation to the discharge in the optic nerve. J. Physiol. **77**: 207.
18. Noell, W. K. 1952. Electrophysiologic study of the retina during metabolic impairment. Am. J. Ophthalmol. **35**: 126.
19. Motokawa, K. & T. Mita. 1942. Über eine einfachere Untersuchungsmethode und Eigenschaften der Aktionsströme der Netzhaut des Menschen. Tôhoku J. Exptl. Med. **42**: 114.
20. Armington, J. C. 1952. A component of the human electroretinogram associated with red color vision. J. Opt. Soc. Am. **42**: 393.
21. Schubert, G. & H. Bornschein. 1952. Beitrag zur Analyse des Menschlichen Elektroretinogramms. Ophthalmologica. **123**: 396.
22. Johnson, E. P. 1949. The electrical response of the human retina during dark adaptation. J. Exptl. Psychol. **39**: 597.

23. Karpe, G. 1945. The basis of clinical electroretinography. Acta Ophthalmol. Suppl. **1**(24): 118.
24. Riggs, L. A. 1954. Electroretinography in cases of night blindness. Am. J. Ophthalmol. **38**: 70.
25. Riggs, L. A. & E. P. Johnson. 1949. Electrical responses of the human retina. J. Exptl. Psychol. **39**: 415.
26. Dodt, E. 1952. Beiträge zur Elektrophysiologie des Auges. II. Über Hemmungsvorgänge in der menschlichen Retina. Arch. Ophthalmol. Graefe's. **153**: 152.
27. Best, W. & K. Bohnen. 1957. Über den "Off-Effekt" in Elektroretinogramm des Menschen. Arch. Ophthalmol. Graefe's. **158**: 568.
28. Dodt, E. 1951. Cone electroretinography by flicker. Nature. **168**: 738.
29. Johnson, E. P. & R. N. Bartlett. 1956. Effect of stimulus duration on electrical responses of the human retina. J. Opt. Soc. Am. **46**: 167.
30. Alpern, M. & J. J. Faris. 1956. Luminance-duration relationship in the electric response of the human retina. J. Opt. Soc. Am. **46**: 845.
31. Johnson, E. P. & T. N. Cornsweet. 1954. Electroretinal photopic sensitivity curves. Nature. **174**: 614.
32. Henkes, H. E., L. H. van der Tweel & J. J. D. van der Gon. 1956. Selective amplification of the electroretinogram. Ophthalmologica. **132**: 140.
33. Armington, J. C. 1955. Amplitude of response and relative spectral sensitivity of the human electroretinogram. J. Opt. Soc. Am. **45**: 1058.
34. Dodt, E. & L. Wadensten. 1954. The use of flicker electroretinography. Acta Ophthalmol. **32**: 165.
35. Iser, G. & G. Goodman. 1956. Clinical studies with flicker electroretinography. Am. J. Ophthalmol. **42**: 227.
36. Granit, R. & A. Wirth. 1953. A scotopic "Blue Shift" obtained by electrical measurements of flicker resonance. J. Physiol. **122**: 386.

SOME ASPECTS OF THE SENSITIVITY OF THE EYE*

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The first experiments reported in this paper deal with human vision. They are related indirectly to a study of visual acuity at low luminances undertaken by Pirenne *et al.* (1957), in which the test objects were black Landolt C's presented in the center of a wide uniform field illuminated with white light. The range of acuity extended from about $\frac{1}{300}$ (gap in the C subtending an angle of $300' = 5°$ at the eye) to $\frac{1}{3}$ (gap in the C subtending $3'$), thus covering scotopic vision and the intermediate region between scotopic and photopic vision. In this range the luminance required to resolve a given C increases steadily as the angular size of the C and of the gap

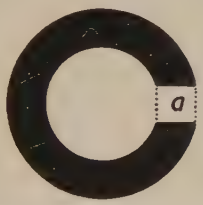

FIGURE 1. Black Landolt C test object, showing the white gap *a*. Reproduced by permission of the Controller of H. M. Stationery Office, from Pirenne *et al.* (1957), p. 43.

a in it (FIGURE 1) both decrease. When the total light flux reaching the eye from gap *a* is calculated at the corresponding threshold luminance, it is found (1) that over most of the above range this flux varies relatively little in absolute value and, further, (2) that the flux is of the same order of magnitude as the lowest flux detectable by the dark-adapted human eye, that is, the flux emitted by a steady effectively point source (TABLE 1). For the absolute threshold of a steady small source of blue-green light (2 mm. artificial pupil; $\frac{1}{4}$ min. exposures, with natural eye movements), recent unpublished experiments give flux values of the order of 120 quanta/sec. at the cornea, thus confirming similar values reported by earlier workers (Pirenne, 1956). The values of TABLE 1 may therefore be somewhat higher than the values of the smallest visible light flux for the same subjects.

S. D. Chalmers (1915) seems to have been the first to reach the general conclusions 1 and 2 mentioned above; however, his quantum computations were not accurate. Chalmers further inferred, as we ourselves also did at first, that the position of the gap in the C was detected by the subject for the

* The apparatus used in the experiments reported in this paper was obtained with the help of research grants from the Nuffield Foundation and the Medical Research Council, London, England.

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seemingly obvious reason that the weak flux of light originating from the white gap *a* was just sufficient to stimulate his retina. In fact, however, the situation may be far more complicated than this.

TABLE 1
FLUX OF BLUE-GREEN LIGHT (0.51 μ) EQUIVALENT TO THE FLUX OF WHITE LIGHT RECEIVED BY THE EYE FROM THE GAP OF A JUST RESOLVABLE LANDOLT C PRESENTED ON A WIDE ILLUMINATED BACKGROUND*

Width of gap in C, minutes of arc	Mean number of quanta per second at the cornea
295	970
191	520
96	260
24	140
12	180
6	650
3	640

* The mean for 20 young subjects, using the natural pupil and ¼-minute exposures (Pirenne *et al.* 1957).

In acuity experiments made with long exposures, the eyes are free to move in a natural manner. Indeed, eye movements are essential. In the scotopic range, steady fixation leads in a few seconds to the subjective blurring of the test object, to its disappearance, and then to disappearance of the whole illuminated field. Presumably these subjective effects occur so readily because the effective retinal stimulus pattern which, of course, consists of quanta absorbed in receptors and is therefore continually fluctuating, is here considerably less sharply defined than it is at high intensities. Slight displacements of the eye will impair relatively slightly the effective stimulus pattern at these levels, whereas movements of the same magnitude must cause significant changes at daylight levels, when the pattern is made up of a much greater number of quanta. To achieve the best acuity at the lower intensities, subjects find that they must move their eyes continually in displacements of a few degrees of arc.

The consequence of such movements, as far as retinal stimulation is concerned, is to make the whole image of the large illuminated field move from place to place on the retina in saccadic fashion. Since the field diameter subtends an angle much larger than the angle corresponding to the saccadic movements, there must nevertheless be a wide retinal area which, apart from physical quantum fluctuations, continues to be illuminated steadily. On the other hand, the eye displacements are large compared with the dimensions of most of the C's; hence, the dark image of the C falls upon a different, that is, a new, part of the retina each time the eye moves into a different position. Before and after receiving the dark image of the C during the corresponding fixation pause, these retinal areas are steadily illuminated.

It may be surmised that it is this sudden dark shadow which, by tem-

porarily cutting off the light from some of the retinal receptors, determines a stimulation pattern in the retina, as a result of which the subject's visual system can resolve the C. One is led to this assumption because the successive retinal areas receiving the image of the white gap *a* are in fact under steady illumination, and steady illumination is known to become ineffective after a few seconds. If this hypothesis is correct, the subject at threshold does see, by a process of direct stimulation, a black ring a part of which is missing. This missing part in the black ring is the gap *a*. However, the subject does *not* see directly the *light* from gap *a*. (I am referring here, not to *slight* displacements of the retinal image, which at high intensities can emphasize contours, but to bodily displacements of the whole image of the test object to fresh parts of the retina. As the number of quanta acting upon the retina is very small in this instance, slight displacements could hardly cause significant changes in the effective pattern of stimulation.)

Attempts to test the latter point were made (Pirenne, 1957) by first placing, in the center of a large field illuminated at an intensity sufficient to allow resolution of the C, an unbroken black ring, instead of the C, and then flashing lights in exposures shorter than the duration of the fixation pauses (0.1 to 0.3 sec.) through the part of the black ring that corresponds to the gap *a* of the C. Under these conditions the threshold for the flash was equal to 4 or 5 times the absolute threshold value—that is, the value obtained with the light of the large field switched off—the flash being presented to the dark-adapted eye with the help of a fixation point. The diameter of the black ring was $1\frac{1}{2}°$, thus corresponding to that of a C of 18′. The field luminance required for resolving such a C is about 100 times that for the absolute threshold of visibility of the large field itself. A field of this luminance was thus sufficient to depress markedly the sensitivity to light flashes of a retinal area surrounded by it. (Clearly a similar test cannot be made using long exposures, for this would simply bring one back not only to the original situation but also to the original problem.) The implication is that the flux from gap *a* in the acuity experiment is probably too weak to stimulate the retina directly.

As the problem of the mechanisms involved in visual acuity is a complex one, it was thought advisable first to study the depressing effect of a dim "surround" on retinal sensitivity. Various experiments were made by flashing light through a hole situated in the center of a wide illuminated field. Except for the fact that the black ring was removed, the conditions were similar to those above, and the results were also similar.

In such experiments the flash of light is not presented as an increment superimposed upon a uniformly illuminated field; it is made, instead, to cover exactly a black patch placed in the middle of a uniform field that thus constitutes, not a background, but a surround to the test stimulus. While this is true of the external experimental arrangements, it may not be so of the retinal stimulus pattern. Light scattering in the eye media, light diffusion in the retina and, in any case, diffraction at the pupil, all will cause some light from the surround to fall into the retinal area corresponding to the geometrical image of the black patch, which coincides with that of the test field.

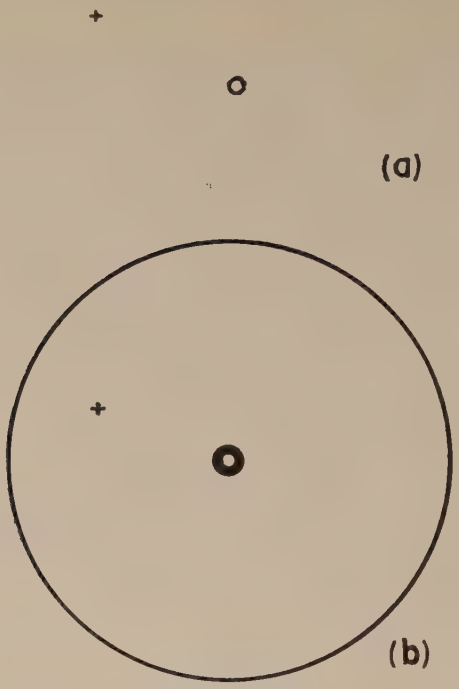

FIGURE 2. Experimental arrangements: (*a*) The small circle (not to scale) represents the test field. The cross on the left indicates the fixation point, placed 10° from the test field. The rest of the visual field is in darkness. (*b*) The test field and the fixation point are in the same positions as in *a*. The test field is placed at the center of an unilluminated circle, which is itself surrounded by a steadily illuminated field 32° in diameter. The region outside this surround is in darkness.

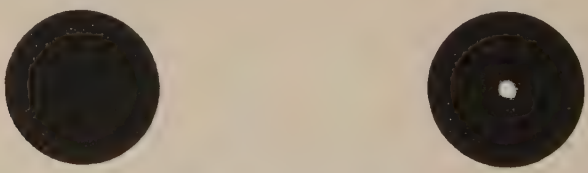

FIGURE 3. The black circle in the center of the steadily illuminated surround before and during a test flash.

Accordingly, experiments were made in which a small test field was separated from the surround by dark zones of various extents. That is, the subject was still presented with an illuminated field having a black spot at its center, but here the test flash filled only part of this black spot (FIGURES 2 and 3). These dark zones constituted rings around the test field but, in contradistinction with those used previously, these rings were, of course, concentric with the test field, and their purpose in the experiment was quite a different one.

In some typical experiments the test flash, 0.1° in diameter and 0.0026 sec. in duration, was presented in the center of a black circle 2° in diameter. The conditions corresponded to the schema of FIGURE 2b. The surround beyond the black ring sent to the eye 2000 quanta of blue-green light (Ilford filter 604) per sec. and per sq. degree, which corresponds approximately to 100 times the absolute threshold for an extended field and long exposures. The center of the test field was placed peripheral to a dim red fixation point by 10°. A 2 mm. artificial pupil and a dental impression to fix the subject's head were used. The black spot became invisible to the subject after he had gazed at the fixation point for a few seconds. This was a test of accurate fixation for, if the eye moved slightly, the black spot was seen again; it ensured that the relevant part of the subject's retina did not receive light directly from the surround because of accidental eye movements. The subject then operated the shutter and reported whether he had seen the flash. The flash intensities were presented at random, and the threshold was estimated by the very convenient and reliable method described in detail by Hartline and McDonald (1957), a method essentially similar to one developed independently by Stiles and Crawford (1934). The threshold value was 250 $h\nu$.

Measurements of the threshold for such a flash were also made with the dark-adapted eye, without using the illuminated surround (FIGURE 2a). This, of course, gave the value of the ordinary absolute threshold for the flash, which was found to be 75 $h\nu$.

Thus, a threshold rise of 0.5 log units was caused by the presence of the surround. This produced a "retinal illumination" corresponding to a mean value of about 0.12 $h\nu$/sec./rod. Since the number of rhodopsin chromophoric groups in a human rod is of the order of 10^8 (Denton and Pirenne, 1954), the percentage of rhodopsin bleached must have been entirely insignificant, even assuming that all the quanta were absorbed and bleached with efficiency unity. In fact, Rushton's (1956) measurements indicate that a proportion absorbed must have been about 0.14; that is, each rod on the average absorbed 1 quantum every minute. It is clear, therefore, that during each experimental trial a proportion of the rods corresponding to the image of the surround must have absorbed no light at all. A fortiori, the stray light falling into the image of the black circle must have been insufficient to deliver even a single quantum to more than a small fraction of the rods covered by it.

However, this does not in any way prove that, weak as it was, the stray light was not responsible for the observed rise in threshold. It is not easy to calculate exactly the intensity of this stray light but, on the basis of Le Grand's (1956) measurements, it can be estimated that at the center of the black circle the stray illumination was of the order of 10 per cent of that in the image of the surround itself. The measurements of Aguilar and Stiles (1954) and of Barlow (1957) show that a background of such intensity (200 $h\nu$/sec. $\times$ degree^2) has a scarcely measurable effect on the increment threshold. I myself also found that such a surround caused but an insignificant rise in threshold in the case of a 0.1° field with a thin black ring around it.

This is strong evidence that the stray light, which in this case indubitably

forms a weak background to the test flash, cannot explain the 0.5 log unit rise in threshold observed when the luminous surround is used. Further evidence is provided by the fact that, when smaller black disks of 1.1° and 0.3° in diameter were used with the same surround luminance, the threshold rise increased only to about 0.6 log units in both cases, hardly a significant change.

The only simple explanation of the results seems to be that the stimulation caused by the luminous surround exerts, through the retina or other parts of the nervous system, an inhibitory influence that acts across regions not directly illuminated upon the visual mechanisms receiving the test flash. There are indications that such inhibition may spread to distances greater than 1 degree, but I have not as yet investigated this point.

It seems plausible that the same type of inhibition will also raise the threshold in the more usual case of a stimulus increment superimposed on the background, for the presence or absence of a luminous background covering the area receiving the test stimulus seems to make no essential difference to the results.

It has been pointed out by Rose (1942) and, independently, by de Vries (1943) as well as Pirenne (1944), that physical fluctuations in the number of quanta received from a luminous background ("noise") should set the limit to the smallest light increment ("signal") detectable in the usual type of intensity discrimination experiments. Provided the relevant parameters relating to temporal and spatial summation in the visual system can be defined safely, statistical considerations should set an impassable theoretical limit to the performance of the eye. The present results, however, suggest that this performance may fall short of the theoretical limit, and thus raise difficulties for theories of intensity discrimination such as Barlow's (1957), in which it is suggested that this ideal level is actually reached.

It is unnecessary to point out that the experiments reported here cannot be explained merely by saying that the eye has become "light-adapted" to the surround, for these reasons: first, the region receiving the flash received but little light from this surround and, second, the problem precisely consists of finding out the nature of light adaptation. The present experiments confirm the view that a nervous inhibitory factor must play a part in such adaptation.

Although I shall not discuss in detail the relevant literature on interaction in the retina or the visual system, I must mention Fry and Alpern's (1953) demonstration of a decrease in the subjective brightness of a test field when an overlapping field is superimposed upon it.

A second series of experiments relevant to the sensitivity of the eye was made on the cat, using the electrophysiological technique of Erulkar and Fillenz (1956). By means of extracellularly placed microelectrodes with tip diameters of 3 to 7μ, they recorded responses from the units of the lateral geniculate body of cats anesthetized with sodium pentobarbital. Further experiments have been made by F. H. C. Marriott, Valerie B. Morris, and myself, partly in collaboration with Erulkar and Fillenz. The electrophysiological technique in our experiments was the same, but we used

a new optical arrangement designed to stimulate a large area of the retina with known amounts of light energy. Erulkar and Fillenz will publish a full account of the general results of their investigations; our results, bearing on absolute threshold values, which are summarized below, will be published separately.

The optical apparatus was arranged to give a Maxwellian field with a diameter about 66° of arc at the eye of the cat. Flashes of 1.1, or about 2 or 3 seconds, were used. The energy of the blue-green light entering the eye and the retinal area illuminated could both be measured, and this made it possible to calculate the threshold energy for the unit in ergs/sec. $\times$ cm.^{2} of retina.

The lowest values of the retinal illumination at which a definite response was obtained were 2.5×10^{-6} and 4.0×10^{-6} erg/sec. $\times$ cm.^{2}. In previous experiments the thresholds for a large field were measured for 22 subjects (Pirenne *et al.*, 1957). Using a 47° field and 15-sec. exposures, the mean value was 9×10^{-8} erg/sec. $\times$ cm.^{2}. It may be concluded that the threshold for the most sensitive units measured was about 30 times the human visual threshold for a similar stimulus, whereas behavioral experiments on the cat show that its visual threshold in terms of retinal illumination is probably about the same as that of man (Pirenne, 1956). The exact reason for this discrepancy is not clear.

The value for man corresponds to a retinal illumination of 1 incident quantum/sec./830 rods (Denton and Pirenne, 1954). The rods of the cat have approximately the same dimensions and distribution as those of the human retinal periphery (see M. Schultze's drawings reproduced in Pirenne, 1948); from this it may be estimated that the lowest threshold value obtained above for the cat corresponds roughly to 1 incident quantum/sec./30 rods. The mean number of quanta absorbed is, of course, lower than this.

In some preparations, the strength of the response in terms of the total number of impulses did not change as much as one might have expected over a supraliminal range of 5 log units. In this connection it must be borne in mind that, even on the extreme assumption that such units would act upon the central nervous system practically in an all-or-none manner, increase in retinal illumination could still be detected owing to the progressive recruitment of units having progressively smaller retinal receptive fields (Pirenne, 1956). High threshold values were often recorded in these experiments; some may have been due merely to the fact that the lateral geniculate body units under the electrode were connected to receptive fields containing only a few rods or cones, instead of thousands of such receptors.

Summary

The threshold of human vision was measured by means of a small, brief flash of blue-green light presented 10° peripherally, under two types of experimental conditions: (1) that corresponding to the absolute threshold, namely, complete darkness except for a dim red fixation point, and (2) with the addition of extended fields of low luminance *surrounding* the test field. Under the latter conditions, even when the surrounds are separated from the

test field by an unilluminated zone their presence can markedly increase the threshold values relative to the values observed under the former conditions. In some cases it can be shown that the stray light falling into the unilluminated zone is probably too weak to cause the observed rise in threshold, which is therefore probably due, not to noise, but to lateral nervous inhibition spreading from the illuminated surround across the dark zone to the retinal region receiving the small flash.

In collaboration, Erulkar, Fillenz, Marriott, Morris, and I obtained electrical recordings from the lateral geniculate body of anesthetized cats (Erulkar and Fillenz, 1956) while a wide retinal area was stimulated with 1.1 or 2- to 3-sec. flashes of blue-green light. The threshold of two of the preparations came down to within 2 log units of that of the human eye. Some of the preparations showed little or no spontaneous activity in the dark. In some cases the strength of the response did not change as much as one might expect over a range of 5 log units above threshold. Increase of luminance may be detected by the CNS owing to the progressive recruiting of units having progressively smaller retinal receptive fields. (Pirenne, 1956.)

References

Aguilar, M. & W. S. Stiles. 1954. Optica Acta. **1**: 59–65.
Barlow, H. B. 1957. J. Physiol. **136**: 469–488.
Chalmers, S. D. 1915. Illum. Engr. London. **8**: 218–220.
Denton, E. J. & M. H. Pirenne. 1954. J. Physiol. **123**: 417–442.
de Vries, H. 1943. Physica. **10**: 553–564.
Erulkar, S. D. & M. Fillenz. 1956. J. Physiol. **133**: 46–47P.
Fry, G. A. & M. Alpern. 1953. J. Opt. Soc. Am. **43**: 189–195.
Hartline, H. K. & P. R. McDonald. 1957. The frequency of seeing at low illumination. Med. Research Council (Brit.) Spec. Rept. Ser. No. **294.**
Le Grand, Y. 1956. Optique Physiologique. **3.** L'Espace Visuel. Editions de la "Revue d'Optique." Paris, France.
Pirenne, M. H. 1944. Proc. Phys. Soc. London. **56**: 354–355.
Pirenne, M. H. 1948. Vision and the Eye. Chapman & Hall, Ltd. London, England.
Pirenne, M. H. 1956. Biol. Revs. Cambridge Phil. Soc. **31**: 194–241.
Pirenne, M. H. 1957. J. Physiol. **137**: 48–49P.
Pirenne, M. H., F. H. C. Marriott & E. F. O'Doherty. 1957. Individual Differences in Night-Vision Efficiency. Med. Research Council (Brit.) Spec. Rept. Ser. No. **294.**
Rose, A. 1942. Proc. Inst. Radio Engrs. **30**: 293–300.
Rushton, W. A. H. 1956. J. Physiol. **134**: 30–46.
Stiles, W. S. & B. H. Crawford. 1934. Proc. Roy. Soc. London. **B116**: 55–102.

RETINAL MECHANISMS FOR CHROMATIC AND ACHROMATIC VISION

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Certain results of our recent studies on what we call the graded photopic response (g.p.r.) of the retina have been presented elsewhere.[1] However, that presentation was limited to a description of the electrophysiological experiments and their results. Our purpose here is to discuss these results in relation to other similar studies[2-16] and to attempt to fit them into the framework of present knowledge of experimental human and animal psychology. This paper first describes the general characteristics of the g.p.r., and then discusses a number of observations in neurophysiological terms. Experimental animal psychology studies on the performance of the visual system of

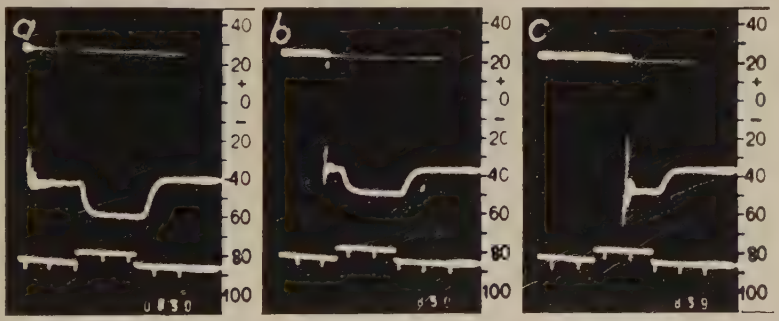

FIGURE 1. Records of the negative resting potential (40 mv) and the response to a light stimulus, which produces an additional (20 mv) increase of negativity. In *a* and *b*, the electrode penetrated the cell membrane just before the light stimulus, and in *c* it penetrated during the light stimulus. Reproduced by permission from Svaetichin.[2]

teleost fishes are reviewed and a comparison is drawn between that system and human vision. The electrophysiological findings on the fish retina are correlated with the observations on vision obtained in training experiments on teleosts and, finally, on the basis of these studies, possible similar mechanisms for chromatic and achromatic vision in teleost and man are discussed.

The graded photopic response. In electrophysiological studies on the isolated teleost retina using small micropipette electrodes, two fundamentally different types of graded electric response to a light stimulus have been described,[1-16] both of which start from a negative membrane potential of about 30 mv (10 to 50 mv), which is the resting potential level in darkness. The response evoked by the light stimulus is either an increase (FIGURE 1) or

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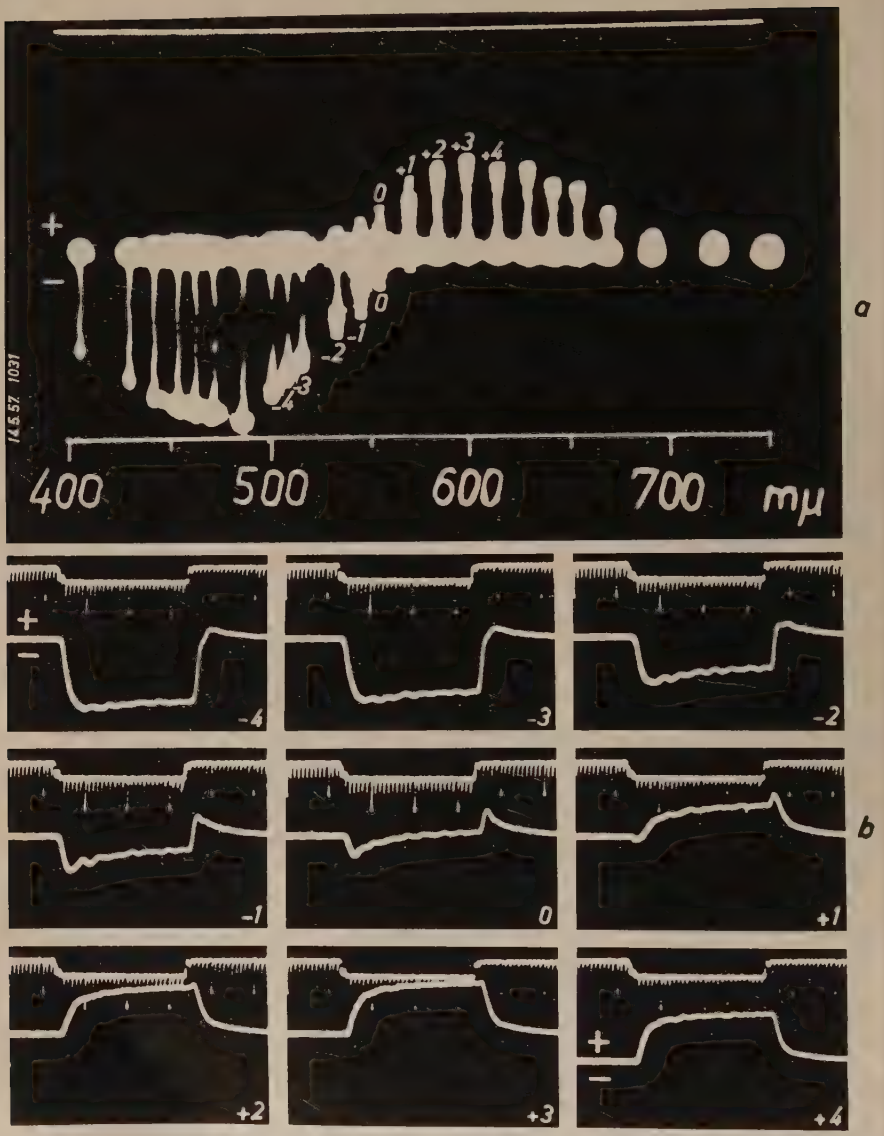

FIGURE 2. (*a*) Peak amplitudes of the responses of the Mugilidae sp. as a function of wave length; (*b*) single-sweep recordings of individual responses. The numbers identify corresponding traces on the record in (*a*). Upper trace indicates duration of flash and time marks 0.1 and 0.01 sec.

a decrease of this negative darkness potential (FIGURE 2) shows both polarities of responses), the new DC level remaining constant for the duration of the light stimulus. This graded response does not obey the "all or none" law, the amplitude of the DC potential changing roughly linearly with the log intensity of light. Furthermore, the response amplitude remains constant as long as the light stimulus of moderate intensity lasts. The "on" latency of the response at 25° C. is about 15 msec. and it varies surprisingly little with the intensity of the light stimulus used; according to Mitari and Yagasaki,[3] the on latency increases up to about 20 msec. at low intensity stimuli and the "off" latency is longer, about 20 to 25 msec. The rise and decay times for a response that reaches the maximal amplitude are each about 50 msec., the

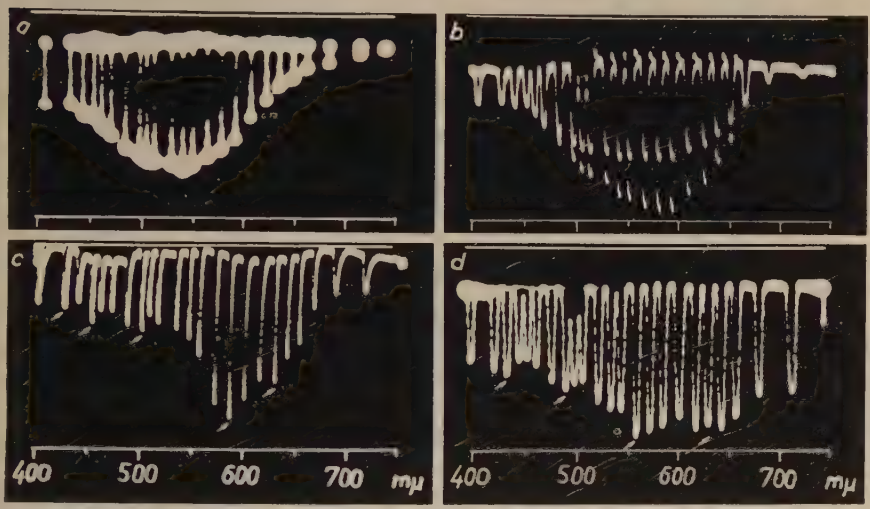

FIGURE 3. Spectral response curves of the L type: *a* obtained from the Lutianidae sp. caught at a depth of 30 to 70 meters; *b*, *c*, and *d* from shallow-water fish. The curves in *b* are from Gerridae sp.; two different recordings of the L curve are superimposed. The curve in *c* is from Mugilidae sp.; in *d* from Centropomidae sp.

time constants increasing when the retina is dark adapted.[3] The accelerating effect of light adaptation on the rise and decay of the responses is striking in studies in which the dark-adapted retina was subjected to a series of repetitive flashes of light.[5]

The types of g.p.r. symbolized by L (luminosity), R-G (red-green), and Y-B (yellow-blue) have been determined on the basis of their reaction to different wave lengths of light. With a largely automatized stimulation and recording system, oscillograms were obtained by us showing: (1) the amplitudes of the g.p.r. as a function of wave length—the spectral response curve—when the retina was subjected to 24 different spectral stimuli of equal energy ranging from 400 to 750 mμ (FIGURE 2*a*); and (2) simultaneously obtained single-sweep recordings showing the time courses of the individual g.p.r. to each of the 24 spectral stimuli (FIGURE 2*b*).

The luminosity response (*L*). The L type of response has always been an increase of the negative resting potential—hyperpolarization—independent of the wave length of the light stimulus used, with the peak of the spectral response curve in the region between 500 and 600 mμ (FIGURE 3 and FIGURE 4*a*). This type of spectral response curve, when recorded from certain species of fishes, had a smooth contour (FIGURE 3*a* and *b*; FIGURE 4*a*-1 and *a*-2; FIGURE 5 and FIGURE 6*f* through *i*). Recordings from other species showed a number of submaxima (FIGURE 3*c* and *d*—arrows). The L response has been obtained from all species of teleost studied. The fish caught at

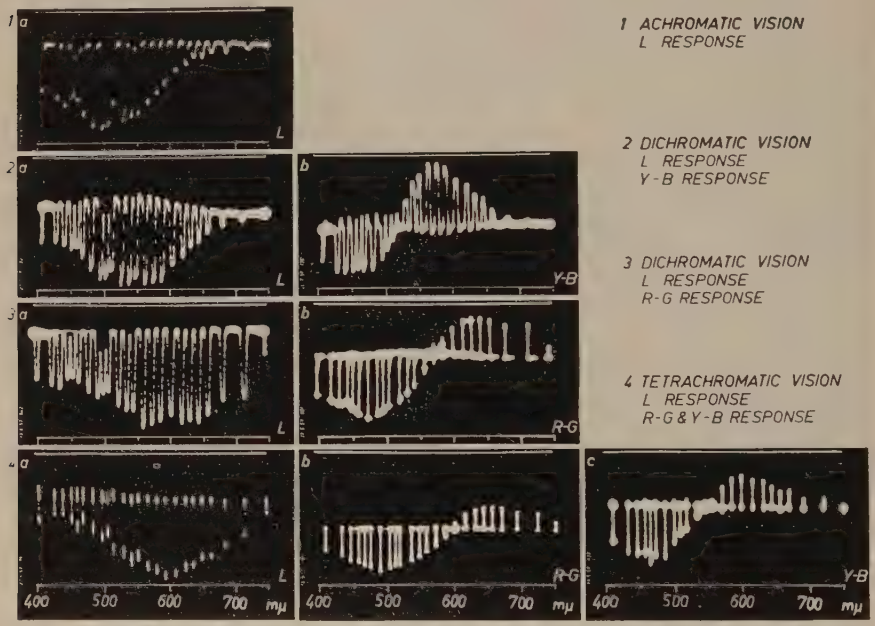

FIGURE 4. Different types of spectral response curve recordings: (1) from Lutianidae sp. at a depth of 30 to 70 meters; (2) from Serranidae sp. at 1 to 2 meters; (3) from Centropomidae sp. at 1 to 2 meters; and (4) from Mugilidae sp. at 1 to 2 meters.

depths of 30 to 70 meters in the Caribbean Sea showed only this type of response, the peak of the spectral response curve being in the green region, between 500 and 530 mμ (FIGURE 3*a* and FIGURE 4*a*-1). On the other hand, the maximum of the L curve obtained from shallow water fishes inhabiting lagoons of the Venezuelan coast was shifted to the yellow-orange region of the spectrum, between 560 and 600 mμ (FIGURE 3*b*, *c*, and *d*, and FIGURE 4*a*-2, *a*-3, and *a*-4).

The chromaticity responses (*Y-B and R-G*). Typical chromaticity responses are shown in FIGURES 2, 4, 5, and 7, and are characterized by having two maxima of opposite polarity. Those we have investigated have shown peaks either in the yellow and blue region of the spectrum, the Y-B responses, or in the red and green region of the spectrum, the R-G responses. In the chro-

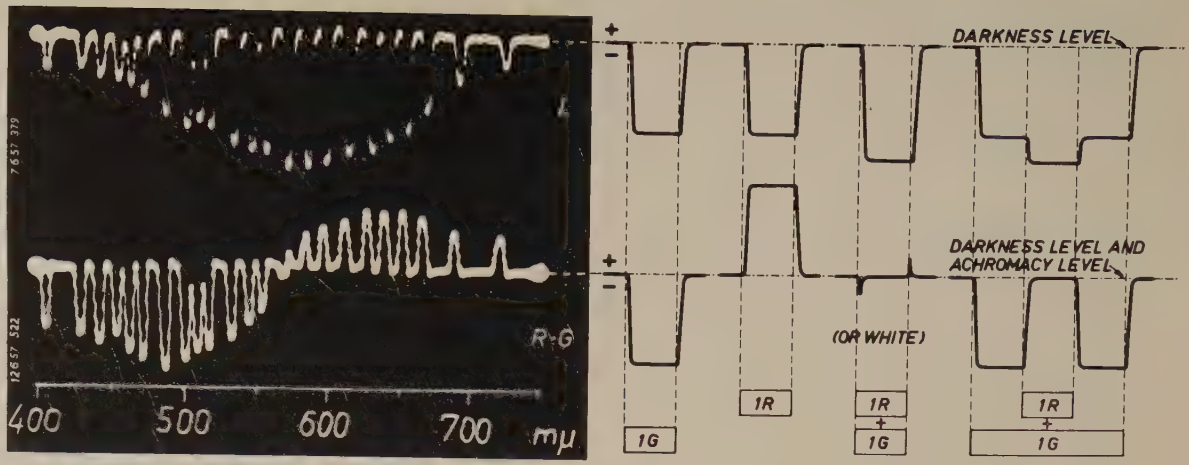

FIGURE 5. Difference between the luminosity and chromaticity responses of the Gerridae sp.

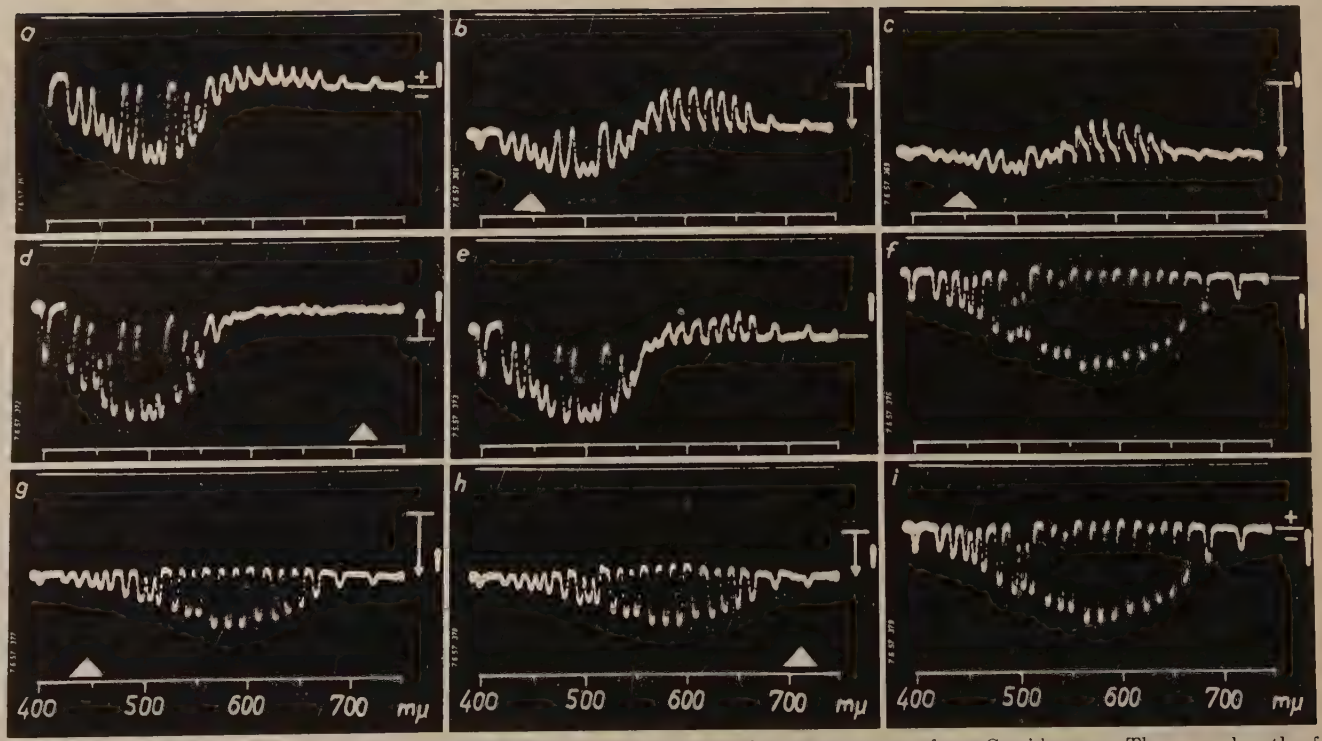

FIGURE 6. Effects of colored background illumination upon the spectral response curves from Gerridae sp. The wave length of background light is indicated by the white triangle.

maticity spectral response curve, one point of the spectrum was neutral; that is, the response to that particular wave length showed no change of the DC potential, while there was a transient deflection of the stimulus at on and at off (FIGURE 2*b*—the response marked zero). The chromaticity responses were generally negative to wave lengths shorter than the neutral point (n.p.), and positive to wave lengths longer than the n.p. The *Mugil* sp. occasionally showed a reversed polarity of the responses. The n.p. of the Y-B response was at about 530 mμ, and the n.p. of the R-G response at about 580 mμ.

FIGURE 7 shows typical spectral-response curve recordings of the Y-B type, for which the electrode was kept in the retina in the same unchanged recording position as that for obtaining curve numbers 134, 135, and 136.

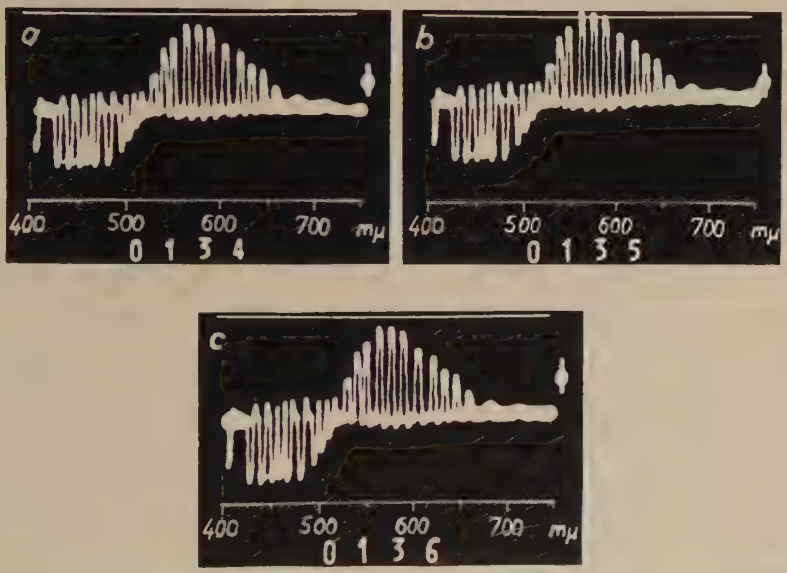

FIGURE 7. Y-B type of spectral response curves obtained in sequence from the same cell. The flattening of the peaks of the spectral response curve in the blue region of the spectrum is due to a saturation effect that is not present when less intense illumination is used.

The subsequent recordings are practically identical; hence the method used gives results that can be duplicated. The resting potential was indicated by the second beam on the oscilloscope and it shows as a spot at the right in each of the recordings in FIGURE 7. Since the distance between the horizontal lines above and below each recording corresponds to 100 mv, the resting potential can be measured, and is about 35 mv; the peak-to-peak response is about 17 mv.

It should be pointed out that Y-B and R-G responses were observed only in the fish caught in shallow water between 1 and 2 meters deep. These fish

also showed the L response. Most of the teleost species studied have shown either the Y-B (FIGURE 4*b*-2) or the R-G (FIGURE 4*b*-3) response, whereas only the *Mugil* sp. exhibits both types of response in the same retina (FIGURE 4*b*-4 and *c*-4).

Generally the amplitude of the resting potential in the L recordings was somewhat lower than that of the Y-B and R-G types, while the L response to a light stimulus showed a somewhat higher amplitude than that of the Y-B and R-G responses. The thresholds were about equal for recordings obtained from the same retina, while the L response might have shown a slightly lower threshold.

Subtraction of opponent responses. The chromaticity responses appear to be combinations of two separate potentials of opposite sign that are somehow "subtracted" from one another. Evidence for this assertion comes from two types of experiment, one showing that the components have different rise times and latencies and the other that they can be abolished selectively by background illumination of appropriate wave length. FIGURES 5 and 6 show typical records of these types of experiments. FIGURE 5*a* is a typical potential versus wave-length record of an R-G response. Each vertical trace was made by exposure to an 0.3 sec. flash of a different wave length. FIGURE 5*b* shows the time courses of the responses to individual flashes, which can be identified by corresponding numbers on the records. It is evident that at wave lengths much shorter than the neutral point the response is a negative, nearly rectangular wave having a short latency and a rapid rise and fall. At wave lengths much longer than the neutral point the responses are still nearly rectangular, but positive in sign and have a longer latency and slower rise and fall. Near the neutral point the responses appear to be made up of the two components added algebraically in different proportions. At the neutral point the steady-state amplitudes of the two components are exactly equal and opposite, so that only the transients at the beginning and end of stimulation appear. Of course, these are due to the difference in the latencies and rise times of the two components.

FIGURE 6 shows the effects of colored background illumination upon typical chromaticity and luminosity responses. The records *a*, *e*, *f*, and *i* are controls without background illumination at the beginning and end of each experiment. In *b*, *c*, and *g*, blue (447 mμ) background illumination was used and in *d* and *h* a red (704 mμ) background was employed. The arrows at the right-hand side of each figure show the DC potential change caused by the background illumination. It is evident that the blue light caused an increased negative "resting" potential, a decrease in the G component of the R-G pair, and an actual enhancement of the R component (possibly due to release of inhibition). Stronger blue light (*c*) practically abolished the G component. With a red background the R component was abolished and the G component enhanced.

Independence of luminosity and chromaticity. When an L response was subjected to a corresponding continuous background light, the general shape of the spectral response curve did not change at all. The curves in FIGURE 6*g* and *h* are identical, although the background light was applied in two different

regions of the spectrum. If the L curve were built up by summation of the individual chromaticity response curves, such a background illumination would be expected to reduce the amplitude of the L response curve selectively in the actual region of the background wave length. Since this was not the case, it is concluded that the teleosts studied thus far have a luminosity mechanism separate from the chromaticity mechanism.

The results are shown schematically in the right-hand portion of FIGURE 5: for the R-G system one unit of green light produces a unit negative response. One unit of red light produces a unit positive response. Equal quantities of red and green light or any amount of white light produce no steady response, but do create "on" and "off" transients, which incidentally may be important in pattern vision. For the L response one unit of red or green light produces a unit negative response and a unit of white light produces a somewhat larger response. However, a unit of red light on a background consisting of a unit of green light, or any stimulus that by itself produces unit response on a background that by itself produces unit response, will produce less than unit response. This is due simply to the nonlinear stimulus-response relationship.

An alternative explanation has been proposed in a personal communication from W. A. H. Rushton, Cambridge University, Cambridge, England, for the constancy of the shape of the L response curve when the retina is illuminated with a steady red or blue light. Rushton suggested that the L response was recorded from a cell supplied with hyperpolarizing synaptic endings from all the chromaticity receptors. On the other hand, the chromaticity response would come, as we have suggested, from a cell supplied with hyperpolarizing and depolarizing endings from an opponent pair of receptors. The major difference between the two systems would be that the chromaticity synapses would be easily fatigued by the background illumination used in our experiment, whereas the L synapses would not. We do not believe that this alternative mechanism is possible because, if two or more receptors of different spectral sensitivity were involved, the nonlinear nature of the response alone would be sufficient to separate the two components. There is no need to assume a fatigable synapse. The L response, like many other electrophysiological potentials, has been shown to vary approximately as the logarithm of the stimulus intensity over a wide range, and to saturate at very high intensities.[2] This effect is supposed to be the mechanism of the familiar Weber-Fechner law of psychology. It has been shown to occur very early in the chain of events initiated in the receptor by the stimulus. For example, the slow potentials recorded from the eccentric cell of the *Limulus* eye also show this quasilogarithmic response.[17]

A consequence of this type of response is that, the greater the background stimulus is, the smaller is the potential produced in response to a constant intensity flash of light. To take a simple example, suppose the L response results from the combination of the responses of a B and a Y receptor. When a background illumination of deep blue light is used, which stimulates the B receptor far more than the Y receptor, the response would be expected to be less vigorous than it would be in the absence of the blue background light,

because the response must start farther up the logarithmic curve. In fact, if the background light were sufficient to push the response to the saturation region of the curve, there would be no response to the test flash. The yellow receptor, however, would be expected to be less sensitive to the deep blue background illumination, so that its response to the constant intensity test flashes would be greater, since its responses start from farther down on the logarithmic curve. The peak of the combined spectral response curve would then shift from an intermediate position between the peaks of the response curves of the two separate receptors toward the peak of the yellow receptor. This did not occur in our experiment. The shape of the curve remained constant regardless of the wave length of the background illumination used. The only effect was a reduction of the sensitivity at all wave lengths. We can think of no other reasonable explanation for this than that whatever pigments are responsible for the L response are combined in a single type of receptor.

It should be mentioned that there is one way in which the response of several receptors could combine to produce the L response. If the receptor process were accurately linear and the postsynaptic mechanism that generates the L response were nonlinear, a background illumination would have no effect on the receptor sensitivity. Preferential stimulation of either receptor would then decrease the amplitude of the L response uniformly at all wave lengths. We do not think this alternative mechanism is likely, since the receptor process is probably not accurately linear.

Hering[18, 19] suggested a separate mechanism for luminosity—the black-white substance—whereas, according to the trichromatic theory of Young-Helmholz,[20] the luminosity information is taken care of by the chromaticity receptors; yet, in order to produce the photopic luminosity curve with its peak at 555 mμ, the degree of contribution to the luminosity of the three receptors must be different! There are numerous psychophysical evidences for a separate luminosity mechanism in man, as well. The totally color-blind "cone monochromat" has a normal luminosity curve like that of fish having only the L response. In general, flicker and fusion are determined solely by the luminosity, being independent of the wave length of light; in heterochromatic flicker photometry the color flicker and the luminosity flicker disappear at different frequencies. These facts are easily explained on the basis of a separate luminosity mechanism with a higher flicker rate, that is, shorter time constants of the g.p.r., than those of the chromaticity mechanisms. It was shown by Pieron[21] and by Wright[22, 23] that in man the effects of chromatic and achromatic adaptation are almost completely independent. A chromatic-adapting light changes the luminosity function, not only in the region of the adapting wave length, but over the entire visible range. This would not be expected on the basis of the trichromatic theory, but it certainly agrees with Hering's proposal[18, 19] and with the experiments on the fish retina described above.

Origin of the g.p.r. In earlier papers by Svaetichin[2, 4] the origin of the g.p.r. was assumed to be the cone myoid and the large synaptic pedicle of the cone. This was also the view held by the other authors concerned with this

potential, with the exception of Tomita,[11] who was of the opinion that the response may not be intracellular and that it is recorded from structures proximal to the receptor layer. By the aid of a marking technique in which crystal-violet stain from the micropipette electrode was moved electrophoretically into the retinal tissue, a spherical dye spot (diam. about 30 μ) was formed at the site from which typical recordings were made.[1, 15] It was demonstrated that the L type of response originated in the outer plexiform layer corresponding to the region of the large horizontal cells and the large cone synapses, whereas the Y-B and R-G types of response were located 20 to 30 μ deeper within the inner nuclear and plexiform layer, in the region of the bipolar and amacrine cells and their synaptic connections to the ganglion cells.

It was concluded that the g.p.r. reflects the activity of the cones only, since this response was regularly found and did not change its character in retinas from which the outer segments and the ellipsoids of the rods had been removed. In this connection it is worth mentioning that von Frisch[24] has demonstrated convincingly that in teleosts, *Phoxinus* sp., the rods are responsible for the achromatic scotopic vision, whereas the cones mediate the chromatic photopic vision. Consequently, we are justified in concluding: (1) that the g.p.r. is a response of the cone system; and (2) that, on the basis of von Frisch's studies, the g.p.r. is actually a photopic response.

Spikes were recorded only when the electrode was inserted deeper into the retina, for the electrode then approached either other types of spike-generating bipolar cells or the ganglion cell layer. No spikes—not even brief injury spikes—were ever seen in connection with the several thousands of recordings of the g.p.r.

It is possible that the g.p.r. is composed of postsynaptic excitatory and inhibitory potentials analogous to the findings of Eccles on the spinal motoneurons.[25] The mechanism responsible for the production of an all-or-none spike recorded from the cell body of, for instance, a motoneuron, functions only within a certain critical range of membrane potential levels, (as shown in FIGURE 20 of the book by Eccles,[25] in which case the range was 42 to 60 mv), whereas the graded postsynaptic potential can be evoked even at resting potential levels close to zero, although the amplitude of the postsynaptic potential is somewhat reduced. Therefore it can be stated in general that the all-or-none spike recorded from the cell body of a neuron is easily blocked when the resting potential is reduced below a critical level, whereas the graded response is more resistant to the resting potential drop caused by the injury from the microelectrode. Since the g.p.r. is a graded response, and perhaps to some extent comparable to the postsynaptic response, one can assume by analogy that the g.p.r. can be evoked even when the membrane potential is considerably reduced. Animals having higher visual acuity than that of the fish also have a greater concentration of neurons per unit of retinal area. From very small neurons, then, we might not be able to record any graded responses with current microelectrode technique. This might explain why we have not been able to obtain any corresponding g.p.r. from the frog retina, whereas it has been obtained from the nocturnal cat.[10, 13, 16]

Ecologic aspects. Everyone who has done skin diving in clear ocean water has noticed its blue-green color at a depth of even a few meters. At greater depths the blue light is completely dominant. Measurements of the solar energy distribution in clear ocean water show that, due to selective absorption in the water, there is a remarkable reduction of the radiant energy, particularly in the long-wave end, but also in the short-wave end of the spectrum. The spectral distribution curves obtained show a steep symmetrical decay at depths of even 20 meters on both sides of the transmission maximum, which is at about 475 mμ. Transmission measurements of the water of the Caribbean Sea off the Venezuelan coast have been reported by Jerlov[26] (see also the review by Clarke[27]). In the waters of lakes, rivers and lagoons, organic and nonorganic material is suspended and dissolved, which alters its absorption characteristics. The lagoons from which the shallow-water fish were obtained for our experiments have water of a yellow-orange color.

The photopic sensitivity maximum of fish might be expected to be influenced by the transmission characteristics of the water they inhabit. It has been shown that the extinction maxima of the extracted—scotopic—rod photopigments of bathypelagic fish are shifted toward the region of about 475 mμ, which is the peak of the solar energy distribution curve at greater depths.[28, 29]

The spectral response curves obtained from fish inhabiting depths of 30 to 70 meters in the Caribbean Sea were without exception of the L type alone, the maximum being at about 500 to 530 mμ, that is, in the green region of the spectrum (FIGURE 3*a* and FIGURE 4*a*-1). The responses of the Y-B and R-G types, which were recorded regularly from the retinas of shallow water species, were never obtained from fish caught at depths of 30 to 70 meters. The spectral response curves of the L type, which always were observed in the retinas of the shallow-water fish in addition to the Y-B and R-G responses, showed a maximum in the region of 550 to 600 mμ, that is, the yellow-orange region of the spectrum.

It seems reasonable to assume from these findings that the L type of response reflects the retinal luminosity mechanism, whereas the Y-B and R-G responses are expressions for the chromaticity mechanism. Color vision would not be of much use to fish inhabiting depths of 30 to 70 meters, since at that depth the illumination is monochromatic, roughly speaking. The L response having its peak value at about 500 to 530 mμ, which is the only type found in fish inhabiting depths of 30 to 70 meters, would correspond rather well to the luminosity function a fish would be expected to possess at these depths. It is no surprise that fish inhabiting these depths have a luminosity mechanism only, and that they are color-blind—cone monochromats, since there would be no selection against this type of mutant.

Moreover, it has been found that the maxima of the photopic luminosity functions obtained in connection with training experiments on shallow-water fish are scattered in the region of 550 to 600 mμ.[30, 31] This corresponds well to the region of the L response maxima recorded from the retinas of shallow-water fish. The location of the luminosity curve maximum agrees well with

the spectral energy distribution of light transmitted through yellow-orange lagoon water. Therefore, all observations agree well with the assumption that the L type of response reflects the retinal luminosity mechanism.

It is also a very likely assumption that the Y-B and R-G responses actually reflect the retinal chromaticity mechanisms, since they were found only in fish inhabiting shallow water, and such fish are generally known to possess color vision.

Training experiments on fish. One can never understand how man actually sees solely on the basis of neurophysiological studies. For this reason, a bridge is required between the psychophysics of vision and the electrophysiological recordings; such a bridge is the experimental study of the psychology of animals.

The vision of teleost fishes has been studied more thoroughly in training experiments than that of any other vertebrate group with the possible exception of the primates, and the results are consistent and clear. The teleosts hitherto studied, primarily the *Phoxinus* sp., have been shown to possess a well-developed color sense.[30, 32-35] It has been possible to train fish to make discriminations independent of brightness among twenty different narrow-band spectral hues.[30] On the basis of the training experiments, a hue discrimination curve for fish was presented, which showed four maxima and a pronounced reduction of hue discrimination ability in the yellow-green region of the spectrum, a curve similar to those of man obtained by Jones[36] and Laurens and Hamilton.[37] Wolff[30] pointed out that fish trained on a particular color remember it for several months.

Purple is an extra-spectral hue that can be created by mixing spectral blue with red. Wolff[30] demonstrated that fish are able also to "synthesize" a hue from blue and red spectral lights, and to distinguish this mixture from red, blue, and any other spectral hue. From this it is clear that fish, as well as humans, have a "closed color circle of Newton," whereby the ultimate ends of the visible spectrum are connected by the purples.

Hamburger[38] trained fish on white light and showed that they were able to distinguish white from any spectral hue. Consequently, for both fish and man, white is a quality of sensation basically different from the sensation of hue. Hamburger also tested the effect on fish of mixing two spectral lights that correspond to a complementary color pair, which gives man the impression of white. The fish were trained to find their food in an area illuminated by white light, and definitely preferred the mixture of the complementary wave lengths (white) to any spectral hue. Moreover, Beniuc[39] has demonstrated the existence of complementary color pairs in fish.

In training experiments on the *Phoxinus* sp., Herter[40-43] proved the existence in fish of both brightness and simultaneous color contrasts. These intriguing phenomena, which are most important for the "dynamic" functional activity of vision, seem to depend on interzonal excitatory and inhibitory activity at the retinal level.

K. von Frisch[24] tested chromatic and achromatic vision of fish trained on color at different levels of light and dark adaptation; he also made parallel morphologic studies on the location of the rods, cones, and pigment cells in

the different states of adaptation—photomechanical movements. Since the fish were trained on color, he was able to determine the level of illumination at which the color vision disappeared. On the basis of these beautiful comparative studies on experimental animal psychology and retinal histophysiology, he gave the most convincing evidence ever presented for the duplicity theory of von Kries, according to which the cones are responsible for color vision and daytime—photopic—vision, whereas the rods mediate the achromatic twilight—scotopic—vision.

This review of teleost vision as revealed by experimental animal psychology studies is far from complete. For instance, all the exciting studies on the space and form sense, flicker fusion, and stroboscopic vision have been omitted. For additional information we refer to the reviews of Walls[44] and Herter.[43] Our purpose has been to emphasize the remarkable similarity between teleost vision and that of man. Of course it is reasonable to expect visual mechanisms among the vertebrates to be based on the same elementary principles. It is impossible to tell whether fish perceive colors in the way we do; the training experiments show only that they are able to discriminate colors, but actually this is sufficient for our purposes.

Psychophysical aspects. Everyone with normal color vision sees the 4 unique hues in the spectrum, blue (B), green (G), yellow (Y), and red (R). Logically, the simplest theory for vision would suggest 4 kinds of receptors corresponding to the 4 different qualities perceived. However, the trichromatic theory proposes 3 different receptors, the only reason being that any hue, or white, can be matched from a minimum of 3 selected spectral lights. According to the trichromatic theory, yellow has no receptor ending of its own, and the sensation of yellow is created by a simultaneous excitation of the red and green receptors. It seems easier to explain the phenomena of color blindness on the basis of opposed R-G and Y-B responses than by the trichromatic theory. A red-green color-blind subject (protanope, deuteranope) sees only 2 colors, the short wave-end of the spectrum being blue and the long wave-end yellow. The correct identification of these colors has been verified by persons with an acquired or unilateral—that is having one normal eye—color blindness.[45, 46]

At certain points of the spectrum 3 of the unique hues are seen as pure qualities, blue at about 470 mμ, green at about 500 mμ, and yellow at about 570 mμ; these points vary somewhat with the state of adaptation. Those with red-green blindness see the long wave-end of the spectrum yellow and the short wave-end blue, with an achromatic, gray neutral point at about 500 mμ, that is, in the region that appears pure green in normal vision! Those with yellow-blue color blindness (tritanope, tetartanope) see the long wave-end of the spectrum red and the middle of the spectrum green, with a neutral point between at about 570 mμ, that is the region that appears pure yellow in normal vision! Further, for the tetartanope the short wave-end of the spectrum is also red, with a neutral point between the red and the green parts at about 470 mμ, that is, the region in which the normal eye sees pure blue! From the spectral response curves of the fish retina, for example, FIGURE 4, and the diagrams in FIGURE 8, it appears that these psychophysical

observations are easily explained on the basis of a subtraction of two different opponent receptor responses.

The neutral point at about 470 mμ seems to be caused by a second submaximum, R_2, of the red component in the short wave-end of the spectrum.

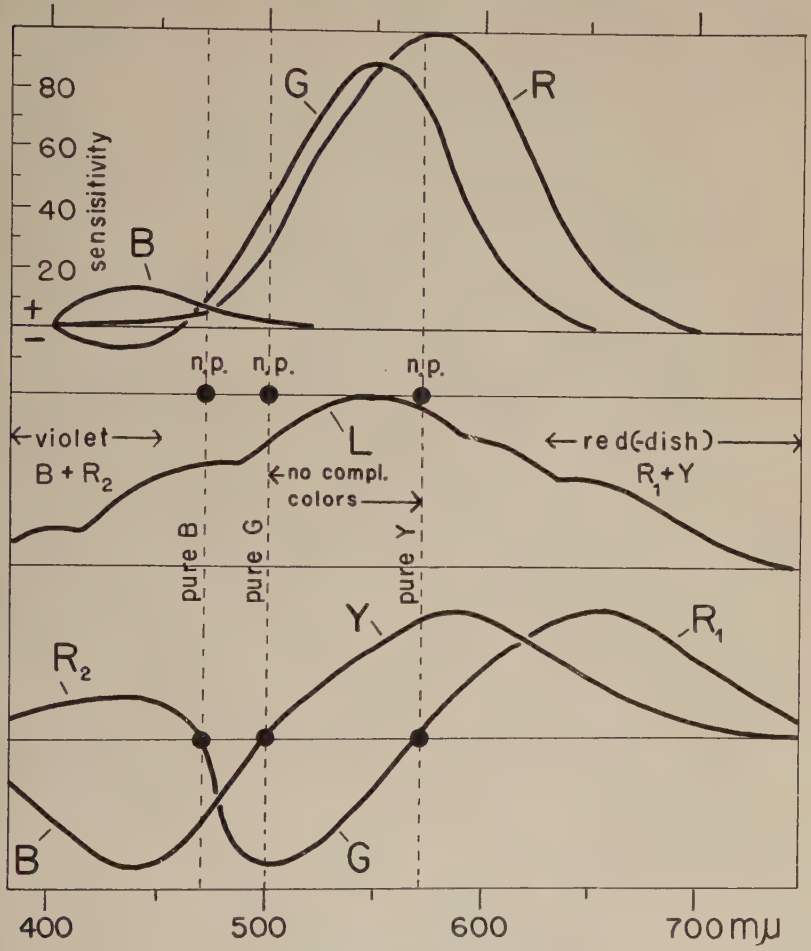

FIGURE 8. Above, a set of trichromatic fundamental response curves;[23] center, a tentative human L type of spectral response curve; below, suggested chromaticity types of spectral response curve in man. The combination of the center and lower curves makes a Hering theory diagram.

There is an indication of an R_2 maximum, as well, in the spectral response curves from certain species of fish.[4, 10] This explains why the short wave-end of the spectrum is violet, a mixture of blue and red, and agrees with the fact that for the tetartanope the short wave-end of the spectrum is red. In the long wave-end of the spectrum the red and yellow spectral response

curves overlap and, since R and Y are additive rather than subtractive components of the same opponent pair, the long wave-end of the spectrum is red and somewhat yellowish.

There are no complementary colors for the spectral lights of the region between the 2 neutral points, 500 mμ and 570 mμ. This fact is also easy to understand from FIGURE 8.

Of the 4 unique hues in the spectrum—B, G, Y, and R—the adjacent ones are psychophysically additive, giving the intermediary hues: B plus G is blue-green, G plus Y is yellow-green, Y plus R is orange, B plus R is blue-red, that is, purple to violet. However, the combinations of B and Y or of G and R, are impossible, since no blue-yellow or green-red hues exist. Thus, psychophysically neither B and Y nor G and R are additive color pairs; rather, they are mutually exclusive, which involves subtraction. If it were possible to obtain Y-B and R-G spectral response curves from the human retina, one might expect them to be qualitatively similar to the lower curves in FIGURE 8. These are almost identical with the curves already suggested on the basis of the Hering opponent color theory.[47]

According to Hering's proposal, the photopic brightness sensation is mediated by a separate luminosity mechanism—black-white substance—as it seems to be in fish. In the scheme indicating the visible spectrum (FIGURE 8, central section) a suggested spectral response curve of the L type in man has been drawn, its maximum situated in the greenish-yellow part, 555 mμ of the spectrum, which is the brightness maximum of the photopic eye. The humps on this luminosity curve are indicated by analogy with the submaxima seen on the L type of spectral response curve recorded from fish. The submaxima on the luminosity curve in FIGURE 8 are located in the regions corresponding to the maxima of foveal "seeing frequency functions" determined by Crozier.[48] The submaxima of the L spectral response curves of fish did not coincide with the chromaticity response maxima; this may be the case for man, as well. Such submaxima might depend, for instance, on the presence of several different photopigments in the luminosity cone.[4]

The pigments combined in the L receptor may be identical with those assumed to exist separately in the chromaticity receptors. That the various submaxima in the L response do not coincide with those of the chromaticity responses is not surprising, since, in general, the peaks obtained by adding or subtracting two overlapping curves do not correspond to the peaks of the curves themselves. It is possible that in hereditary color blindness the gene required for synthesizing a particular pigment is missing. The pigment would then be absent in the luminosity as well as the chromaticity system which would account for the loss of brightness sensitivity[46] in the appropriate part of the spectrum observed, as well as the loss of color vision. It can be assumed that in protanopia the "red" pigment is missing, and in deuteranopia the "green" photopigment is absent. If the other counterbalance is absent from a balanced opponent color system, it is reasonable to suppose that the paired mechanism is out of function. Hence, if either the red or the green receptor component is missing, a red-and-green color blindness is a likely consequence. If we assume that in protanopia the "red" photo-

pigment is lacking, we may assume that the photopigments with maximal absorption in the long wave-end of the spectrum will also be absent from the luminosity cones of a protanope. A corresponding reasoning can be applied in deuteranopia, assuming that the "green" photopigment is absent. If some of the presumably 5 or 6 photopigments are lacking in the luminosity cone, an alteration of the peak, the shape, and the absolute sensitivity of the luminosity function can be expected.

Pattern and diffuse vision. Any theory of vision must consider the dynamic aspects of vision revealed by highly interesting experiments by Ratliff,[49] Ditchburn and Ginsborg,[50] Riggs *et al.*,[51, 52] Ditchburn and Fender,[53] and Ditchburn.[54] This work shows that under the conditions of an artificially stabilized retinal image the pattern vision fails and there are also severe disturbances of the perception of hue and saturation. The observations indicate the existence of adaptation phenomena in the nervous communication mechanisms of the cone system, possibly the ganglion cells. White light evoked on-off deflections of the chromaticity type of response. This on-off pattern may be important for visual acuity and contrast phenomena. It seems likely that the paired chromaticity mechanisms are responsible for pattern vision in chromatic and achromatic conditions, whereas the luminosity function represents a diffuse system in the retina.

Relation of the g.p.r. to negative afterimages and adaptation. Although the amplitude of the g.p.r. does not change with time during prolonged illumination of moderate intensity, it does decrease with time if a very strong illumination is used. Furthermore, the potential does not return to the initial resting value—darkness level—when the light is turned off; instead, there is an overshoot in the potential that persists for a considerable time. If the process that generates the g.p.r. governs the signals in the visual pathway, the overshoot would be expected to represent an effect opposite to the previous one, which decreases with time. This could be the mechanism involved in the production of negative afterimages.

Furthermore, the gradual decrease of the response amplitude when strong illumination is used may correspond to the slow adaptation process observed in photopic vision in man, whereas, the fast, or alpha, adaptation may be related to the rise time of the g.p.r., which is about 50 msec. in fish.

Receptor process—postsynaptic process. Whatever neurophysiological interpretation and view of the origin of the g.p.r. are eventually accepted, the intraretinal potential changes presented above cannot be neglected. If we assume that similar potentials are transmitted as quantities, for example as changes in frequency of discharge of the ganglion cells (both polarities must be transmitted as quantities in addition to reciprocal excitatory-inhibitory effects between them), to the optic centers in man, they would satisfy the basic requirements on signals for a photopic visual system.[55] Studies on a quantified opponent color theory have been carried out by Jameson and Hurvich,[56-60] v. Tschermack-Seysenegg,[47] and Linksz.[61]

It is important to emphasize that our experiments do not furnish direct evidence of the nature of the receptor cells themselves, since the responses were shown to have been picked up from more proximal layers of the retina.

The chromaticity responses are thought to be combinations of responses of receptor cells having different spectral sensitivities. Therefore, we do not know how many types of receptor cells there are, or the nature of their individual spectral response curves. We know only that the responses of opponent pairs of receptors are in some way combined, and that at least 3 distinct types of chromaticity receptors must exist in a retina that gives both R-G and Y-B responses. It is possible that there are 4 or even more receptors, but 3 connected in pairs seem sufficient to give the responses found in the *Mugil* retina. Thus, it is entirely possible that this retina is trichromatic at the receptor level and tetrachromatic at the postsynaptic level and in the visual pathways, as has been suggested by Adams[62, 63] and by Müller.[64, 65]

An interesting example of such an arrangement is found in the color television system currently in use in the United States.[66] In this system light from the scene being televised is split into red, green, and blue components, which are then imaged on separate camera tubes. The signals from the separate tubes are combined to form a luminosity signal known in the industry as the Y signal. This signal is transmitted directly as video information and can be picked up on black-and-white receivers without modification. In addition, the Y signal is subtracted from the R and B signals to give R-Y and B-Y components, which are transmitted separately. In the receiver, the R-Y, B-Y, and Y components are combined in such a way that the original R, G, and B signals are reconstituted. These are applied to separate electron guns in the picture tube which illuminate separate R, G, and B phosphors. A stranger analyzing the system would conclude that it was a Young-Helmholz device if he examined the camera tubes and the picture tube, and a Hering device if he examined the transmission pathways. Investigators studying the eye may find themselves in exactly the same position. Before a complete theory of color vision is possible, it is essential that we find a means of studying the activity of the receptors themselves.

References

1. MacNichol, E. F. & G. Svaetichin. 1958. Symposium on electrophysiology of the visual system. Natl. Inst. Health. Bethesda, Md. Am. J. Ophthalmol. **46**: 26(1958).
2. Svaetichin, G. 1953. Acta Physiol. Scand. **29.** (Suppl.) **106**: 565.
3. Mitari, G. & Y. Yagasaki. 1955. Ann. Rept. Research. Inst. Environment. Med. Nagoya Univ. : 54.
4. Svaetichin, G. 1956. Acta Physiol. Scand. **39.** Suppl. **134**: 17.
5. Svaetichin, G. 1956. Acta Physiol. Scand. **39.** Suppl. **134**: 47.
6. Svaetichin, G. 1956. Acta Physiol. Scand. **39.** Suppl. **134**: 55.
7. Svaetichin, G. 1956. Acta Physiol. Scand. **39.** Suppl. **134**: 67.
8. Svaetichin, G. 1956. Acta Physiol. Scand. **39.** Suppl. **134**: 93.
9. Svaetichin, G. & R. Jonasson. 1956. Acta Physiol. Scand. **39.** Suppl. **134**: 3.
10. Motokawa, K., T. Oikawa & K. Tasaki. 1957. J. Neurophysiol. **20**: 186.
11. Tomita, T. 1957. Japan. J. Physiol. **7**: 80.
12. Tamura, T., G. Mitari & Y. Sugita. 1957. Bull. Japan. Soc. Sci. Fisheries. **23**: 86.
13. Grüsser, O. J. 1957. Naturwiss. **44**: 522.
14. Jung, R., O. Creutzfeldt & O. J. Grüsser. 1957. Deut. Med. Wochschr. **82**: 1050.

15. MacNichol, E. F., L. Macpherson & G. Svaetichin. 1957. Symposium on visual problems of colour. Natl. Phys. Lab. Teddington, England. Paper **39.**
16. Brown, K. T. & T. Wiesel. 1958. Symposium on the electrophysiology of the visual system. Natl. Inst. Health. Bethesda, Md. Am. J. Ophthalmol. In press.
17. MacNichol, E. F. 1956. Visual receptors. Biological transducers. Am. Inst. Biol. Sci. Washington. **1**: 34.
18. Hering, E. 1875. Sitzber. Kaiser. Akad. Wiss. Wien. Math. Natur. Kl. **3**: 70.
19. Hering, E. 1920. Grundzüge der Lehre vom Lichtsinn. Berlin, Germany.
20. Helmholz, H. von. 1911. Hdb. physiol. Optik. 3d. ed. Voss. Leipzig, Germany.
21. Pieron, H. 1952. The Sensations. New Haven, Conn.
22. Wright, W. D. 1934. Proc. Roy. Soc. London. **B115**: 49.
23. Wright, W. D. 1946. Researches on Normal and Defective Colour Vision. London, England.
24. Frisch, K. von. 1925. Z. vergleich. Physiol. **2**: 393.
25. Eccles, J. C. 1957. The Physiology of Nerve Cells. Baltimore, Md.
26. Jerlov, N. G. 1951. Reports of the Swedish Deep-Sea Expedition 1947–48. III. Physics and Chemistry. Fasc. **1**: 1. Elanders. Göteborg, Sweden.
27. Clarke, G. E. 1954. Elements of Ecology. New York, N. Y.
28. Denton, E. J. & F. J. Warren. 1956. Nature. **178**: 1059.
29. Munz, F. W. 1957. Science. **125**: 1142.
30. Wolff, H. 1925. Z. vergleich. Physiol. **3**: 279.
31. Grundfest, H. 1932. J. Gen. Physiol. **15**: 507.
32. Frisch, K. von. 1913. Zool. Jahrb. Abt. Allgem. Zool. Physiol. Tiere. **34**: 43.
33. White, G. M. 1919. J. Exptl. Zool. **26**: 443.
34. Reeves, C. D. 1919. Behav. Monogr. **4** (3): 1.
35. Schiemenez, F. 1924. Z. vergleich. Physiol. **1**: 175.
36. Jones, L. A. 1917. J. Opt. Soc. Am. **1**: 63.
37. Laurens, H. & W. F. Hamilton. 1923. Am. J. Physiol. **65**: 547.
38. Hamburger, V. 1926. Z. vergleich. Physiol. **4**: 286.
39. Beniuc, M. 1933. Z. vergleich. Physiol. **18**: 437.
40. Herter, K. 1930. Z. vergleich. Physiol. **11**: 730.
41. Herter, K. 1950. Moderne Biologie. Festschr. Nachtsheim. Berlin. : 283.
42. Herter, K. 1950. Biol. Zentr. **69**: 283.
43. Herter, K. 1953. Die Fischdressuren und ihre sinnesphysiologischen Grundlagen. Berlin, Germany.
44. Walls, G. L. 1942. The Vertebrate Eye and Its Adaptive Radiations. Michigan.
45. Judd, D. B. 1948. J. Research. Natl. Bur. Standards. **41**: 247.
46. Graham, C. H. & Y. Hsia. 1958. Science. **127**: 575.
47. Tschermack-Seyssenegg, A. v. 1952. Introduction to Physiological Optics. Thomas. Springfield, Ill.
48. Crozier, W. J. 1950. J. Gen. Physiol. **34**: 87.
49. Ratliff, F. 1952. J. Exptl. Psychol. **43**: 163.
50. Ditchburn, R. W. & B. L. Ginsborg. 1952. Nature. **170**: 36.
51. Riggs, L. A., F. Ratliff, J. C. Cornsweet & T. N. Cornsweet. 1953. J. Opt. Soc. Am. **43**: 495.
52. Riggs, L. A., J. C. Armington & F. Ratliff. 1954. J. Opt. Soc. Am. **44**: 315.
53. Ditchburn, R. W. & D. H. Fender. 1955. Optica Acta. (Paris). **3**: 128.
54. Ditchburn, R. W. 1956. Research. **9**: 466.
55. Macadam, D. L. 1957. Symposium on the visual problems of colour. Natl. Phys. Lab. Teddington, England. Paper **25.**
56. Jameson, D. & L. M. Hurvich. 1955. J. Opt. Soc. Am. **45**: 546.
57. Hurvich, L. M. & D. Jameson. 1955. J. Opt. Soc. Am. **45**: 602.
58. Jameson, D. & L. M. Hurvich. 1956. J. Opt. Soc. Am. **46**: 405.
59. Hurvich, L. M. & D. Jameson. 1956. J. Opt. Soc. Am. **46**: 416.
60. Hurvich, L. M. & D. Jameson. 1957. Symposium on the visual problems of colour. Natl. Phys. Lab. Teddington, England. Paper **22.**

61. Linksz, A. 1952. Physiology of the Eye. New York, N. Y.
62. Adams, E. Q. 1923. Psychol. Rev. **30**: 56.
63. Adams, E. Q. 1942. J. Opt. Soc. Am. **32**: 168.
64. Müller, G. E. 1924. Darstellung und Erklärung der verschiedenen Typen der Farbenblindheit. Göttingen, Germany.
65. Müller, G. E. 1930. Z. Psychol. Ergzb. **17–18**: 46; 508.
66. Hershey, R. D. 1956. Color Television—Simplified Theory and Servicing Techniques. Philco Corporation. Philadelphia, Pa.

ANALYSIS OF RECEPTIVE FIELDS IN THE CAT'S RETINA

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This is a report on current work concerning functional organization of the receptive field. The receptive field of a ganglion cell was defined by Hartline (1938) as the retinal area from which it is possible to evoke a discharge by light stimulation. Kuffler (1953) showed that in the cat the receptive field in the light-adapted state is organized in the following manner: there is either an "on" center and "off" periphery, or vice versa. He also demonstrated that the central and peripheral regions interact and that they are mutually inhibitory.

The present study was made with the closed-eye technique and multibeam ophthalmoscope developed by Talbot and Kuffler (1952). This made it possible to record from the retina with intact optics and under normal physiological conditions of the eye. The technique also permitted stimulation of the retina with well-focused light spots, and both the electrode and the stimulus spot were localized under visual observation. The cats were maintained under light barbiturate anesthesia, immobilized by continuous intravenous infusion of succinylcholine, and artificially respirated. Unilateral pneumothorax was used to minimize respiratory movements of the retina. Such a preparation can be kept in good condition for over 24 hours. Micropipette electrodes were used with tip diameters of less than 0.5 μ. Stable extracellular recordings have been made from single ganglion cells for as long as four hours.

In the light-adapted state the receptive fields of all ganglion cells were found to have the same general organization described by Kuffler (1953); that is, the central and peripheral zones responded to light stimulation with opposite discharge types. Further information about the functional organization of the receptive field can be obtained with area-threshold measurements, as shown by Barlow (1953) in the frog and by Barlow *et al.* (1957) in the cat. Area-threshold curves in the cat show an initial decrease of threshold as the stimulus area is increased, indicating area summation for the center-type response. With further increase in spot size, the peripheral portion of the receptive field comes to be included in the stimulated area, and the threshold rises, due to peripheral inhibition of the center-type response. In this study the threshold for each stimulus area was determined as the lowest intensity of a 0.8 sec. light stimulus that gave a clear change of the maintained discharge rate. Quantitative differences between the area-threshold curves of different ganglion cells have been observed. These differences indicate that receptive fields of ganglion cells vary both in the area of summation for the center-type response and in the strength of peripheral suppression of the center response. Generally the ganglion cells having a small center area, as measured by summation of the center-type response, were strongly suppressed by stimulation of the periphery. Thus a

small light spot of 0.8 sec. duration and covering only the center of the receptive field may have a threshold as much as 2 log units lower than the threshold for a large spot covering the entire receptive field. Ganglion cells that exhibit summation over a larger central area show less inhibition from the periphery. There is also a clear tendency for the receptive fields in the area centralis to have smaller areas of summation for the center-type response than the receptive fields of ganglion cells have in the peripheral retina. It seems likely that the specialized receptive fields of ganglion cells in the area centralis are functionally related to high visual acuity in this part of the retina.

It has been reported by Barlow *et al.* (1957) that near the termination of dark adaptation a change occurs in the functional organization of the receptive field. In the dark-adapted state, the center-type response is evoked from the entire receptive field, which is therefore uniform, with no sign of peripheral inhibition. The dropping out of inhibition from the periphery seems to provide a basis for the larger area of summation for the center-type response, which should increase the sensitivity for large stimuli. Thus Barlow and his co-workers suggest that the change to a uniform receptive field provides one of the mechanisms for the increase in sensitivity during dark adaptation. In this study a change in functional organization of the receptive field occurred quite frequently during the early phase of dark adaptation. This finding suggests the possibility that the rapid increase of sensitivity during the early part of dark adaptation is due, at least partly, to a change of neural organization. Further studies are required concerning the time course of the change in receptive field organization in relation to the increase of sensitivity during dark adaptation.

References

Barlow, H. B. 1953. Summation and inhibition in the frog's retina. J. Physiol. **119**: 69–88.

Barlow, H. B., R. FitzHugh & S. W. Kuffler. 1957. Change of organization in the receptive fields of the cat's retina during dark adaptation. J. Physiol. **137**: 338–354.

Hartline, H. K. 1938. The response of single optic nerve fibers of the vertebrate eye to illumination of the retina. Am. J. Physiol. **121**: 400–415.

Kuffler, S. W. 1953. Discharge patterns and functional organization of mammalian retina. J. Neurophysiol. **16**: 37–68.

Talbot, S. A. & S. W. Kuffler. 1952. A multibeam ophthalmoscope for the study of retinal physiology. J. Opt. Soc. Am. **42**: 931–936.

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